Annexinopathies:
physiological significance of endogenous Annexin A5 in human pathology

Larissa Hiddink-Emelianova

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Larissa Filaretovna Hiddink-Emelianova
geboren op 19 januari 1968
te Izhevsk, Rusland

Promotor
Prof. dr. J.H. Jansen

Copromotor
Dr. W.L. van Heerde

Manuscriptcommissie
Prof. dr. G.A.P.J.M. Rongen (voorzitter)
Prof. dr. C.P. Reutelingsperger (Maastricht University)
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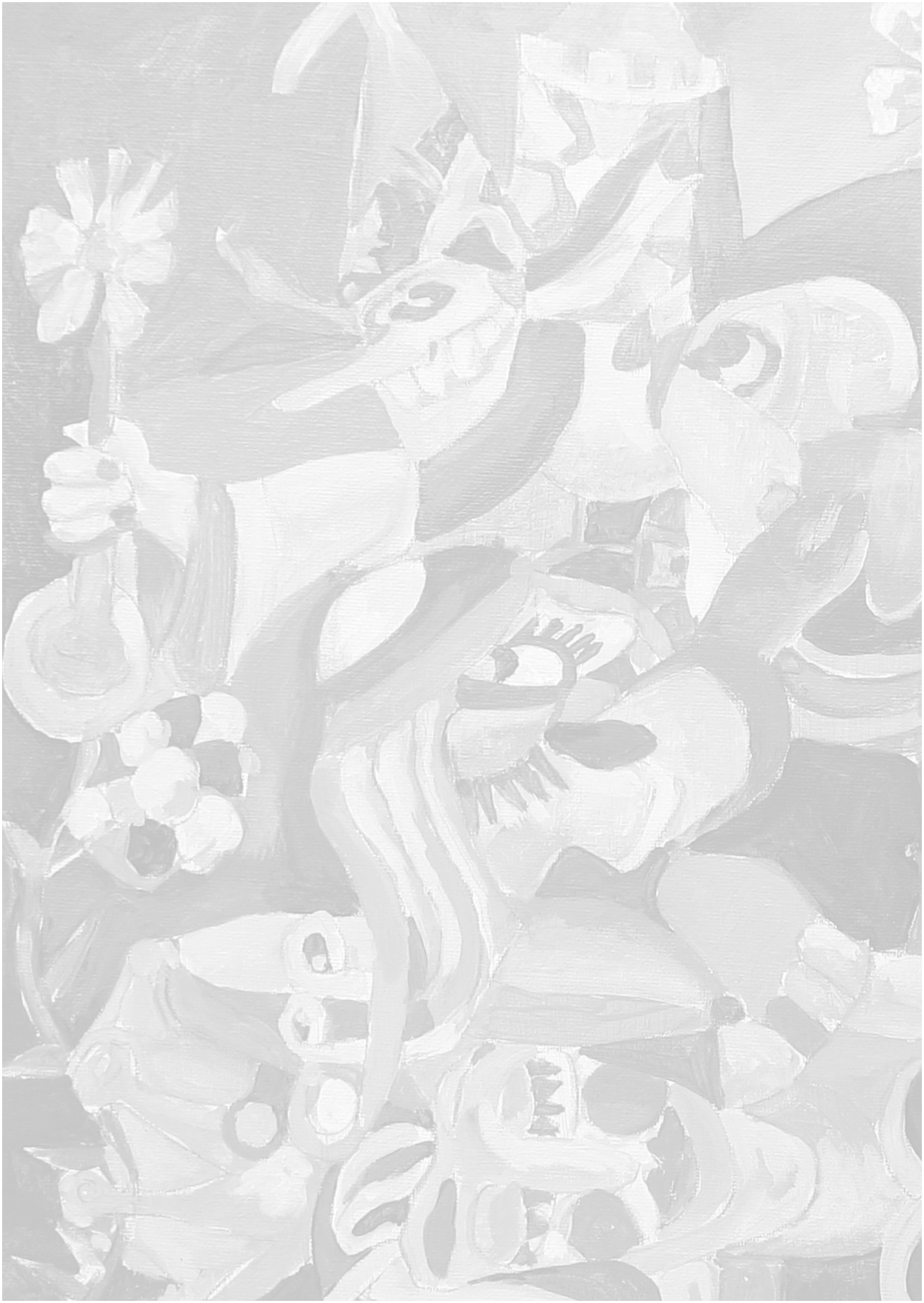

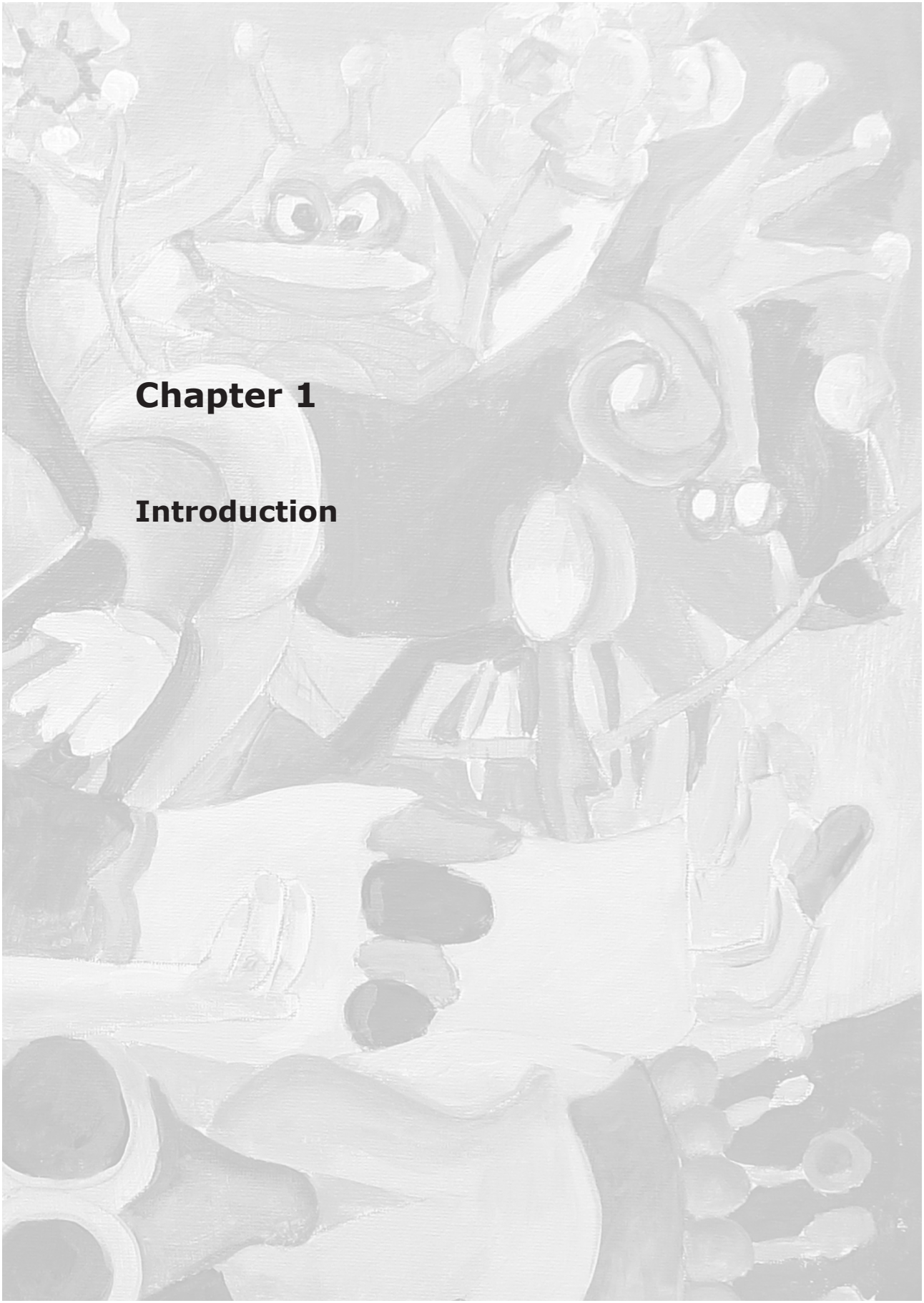

Chapter 1

Introduction

THE ANNEXINS

The annexins (from the Greek annex - "bring/hold together") are a family of more than 160 membrane-binding proteins that share structural properties and biological activities associated with membrane-related processes [1;2]. Annexin proteins are expressed in more than 65 different groups of organisms ranging from fungi and protists to plants and higher vertebrates [1;3]. In humans (the annexin A group), 12 annexins have been identified (i.e., Annexin A1 (ANXA1) - ANXA11 and ANXA13). The members of the annexin family have two important common criteria. First, annexin proteins share a highly homologous core domain, comprising four so-called annexin repeats of approximately 70 amino acids long (with the exception of ANXA6, which consists of 8 annexin repeats), packed into an α-helical disk [1]. The ability of annexins to bind to negatively charged phospholipids in a Ca^{2+}-dependent manner is the second common characteristic of proteins within the annexin family. Annexin proteins participate in a variety of membrane-related processes, both inside the cell (e.g., signal transduction, endocytosis, cytoskeleton organization, cellular proliferation, differentiation) and in the extracellular environment (e.g., receptors for serum proteases, blood coagulation, inhibitors of neutrophil migration) [2]. However, their physiological function remains largely unknown. In 1999, *Jacob H. Rand* introduced the term "annexinopathies" [4]. It has been shown that the abnormalities in the expression levels of two annexins contribute to pathogenesis of human diseases: overexpression of ANXA2 on leukocytes is associated with increased fibrinolysis and bleeding in acute promyelocytic leukemia [5]; reduced expression of ANXA5 on placental syncytiotrophoblasts and endothelial cells is linked to pregnancy loss and to thrombosis in the antiphospholipid syndrome [6-8].

This thesis focuses on ANXA5, the most abundant protein of the annexin family and a physiological anticoagulant protein. Two main aspects were of interest. First, the mode of action of ANXA5-based anticoagulation on cell surfaces exposing negatively charged phospholipids. The second aspect of our interest was the associations between genetic variations in the *ANXA5* gene and diseases related to thrombosis and inflammation. This general introduction starts with an overview of hemostasis linked to our first question. In regard to the second aspect of this thesis, the information about the structure of the *ANXA5* gene and *ANXA5* polymorphic variations will be presented later in the introduction chapter.

GENERAL OVERVIEW OF HEMOSTASIS

The hemostatic system is a highly complex process that maintains blood in a fluid state under physiological conditions and even after the vascular injury [9;10]. The interactions between the endothelial cells lining the vessel wall, blood flow and blood coagulability (i.e. procoagulant and anticoagulant factors, fibrinolysis) play an important role in the hemostatic balance. An impaired equilibrium between the components of the hemostatic system may result in either thrombotic complications or a bleeding tendency. Knowledge of the hemostatic system is important for a better understanding of disease states associated with thrombosis (i.e., the formation of a clot within a blood vessel), such as venous thromboembolism and atherothrombosis (thrombosis triggered by plaque rupture, leading to myocardial infarction and stroke) [9;11;12].

Hemostasis can be divided into primary hemostasis (the formation of the platelet plug), secondary hemostasis (coagulation) and tertiary hemostasis (fibrinolysis). Upon vascular damage, the contraction of the vessel wall occurs to reduce blood flow through the vessel wall rupture. Platelets adhere to the subendothelial matrix, become activated, and form the primary hemostatic plug [13]. Plasma factor (F) VII/VIIa binds to extravascular tissue factor (TF), initiating thereby the coagulation protease cascade that in turn leads to the formation of thrombin. Activation of platelets results in the translocation of phosphatidylserine (PS) from the inner leaflet of the plasma membrane to the outer leaflet [14] that also promotes the enzymatic reactions of the coagulation cascade. Thrombin, the key enzyme in hemostasis, activates platelets, FV and FVIII, FXI, FXIII, TAFI (thrombin activatable fibrinolysis inhibitor) and cleaves plasma fibrinogen into fibrin. The fibrin network stabilizes the plug formed by aggregated platelets. Once the vessel wall is repaired, the fibrin platelet clot will be lysed (fibrinolysis) in order to restore the normal blood flow.

Blood coagulation (**Figure 1**) as a cascade or waterfall of consecutive enzymatic reactions (a cascade model) has been described 51 years ago [15;16]. According to that model, clotting factors, which are present in blood as zymogens, are converted into active enzymes via either the extrinsic or intrinsic pathways. The extrinsic pathway of blood coagulation is initiated by TF present on the surface of perivascular tissue, which binds circulating FVII/activated FVII (FVIIa). The TF/FVIIa complex is the physiological activator of the coagulation reactions [2], and this complex can activate FIX and FX. FXa assembled with the non-enzymatic cofactor FVa on an anionic phospholipid surface in the presence of Ca^{2+}-ions (prothrombinase complex) leads to the formation of a small amount of thrombin. An alternative way of thrombin generation occurs via the intrinsic pathway or contact activation pathway when blood is exposed to an exogenous negatively charged surface (collagen, glass, kaolin). Contact activation leads to autoactivation of FXII, which in turn activates FXI. Thereafter, FXIa activates FIX. FIXa associates with the non-enzymatic protein FVIIIa on a negatively charged phospholipid

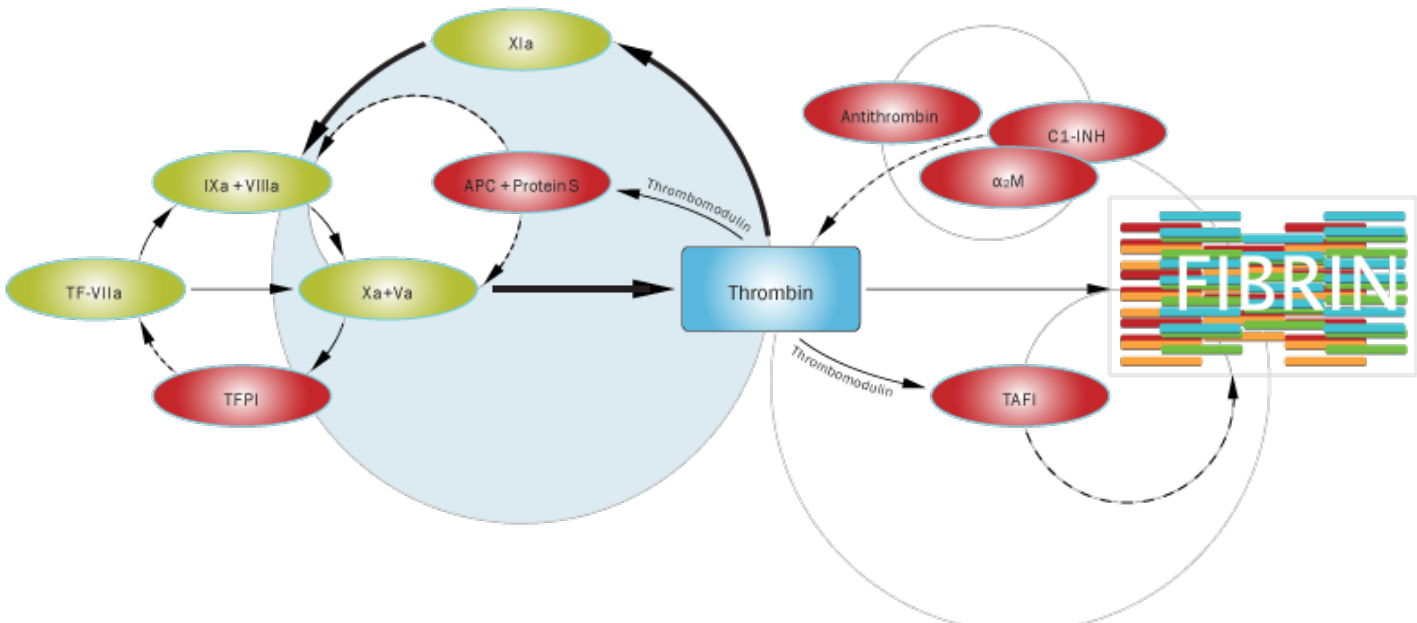

Figure 1. Coagulation cascade. Adapted from the thesis "Simultaneous thrombin and plasmin generation" written by H.A. van Geffen, 2012; with permission of H.A. van Geffen. Abbreviations: TF, tissue factor; TFPI, tissue factor pathway inhibitor; APC, activated Protein C; C1-INH, C1-inhibotor; α_2M, α_2-macroglobulin; TAFI, thrombin activatable fibrinolysis inhibitor. Green color indicates factors with procoagulant activities; red color – factors with anticoagulant activities.

surface in the presence of Ca^{2+}-ions to form the tenase complex. The tenase complex activates FX, which in turn rapidly converts prothrombin into thrombin. A secondary burst (propagation) of thrombin formation necessary for an adequate hemostatic response is provided by the activation of FXI by thrombin (the positive feedback in the coagulation cascade) [17]. Platelets play a critical role in the amplification of the coagulation cascade by providing a catalytic surface [10;18]. Thrombin cleaves soluble fibrinogen into insoluble fibrin polymers, which stabilize the platelet plug.

In the late 1990s, a revised model of blood coagulation was postulated in which there is no distinction between the extrinsic and intrinsic pathways [19]. Coagulation reactions occur on a phospholipid surface containing anionic phospholipids, especially PS, often provided by activated platelets, activated endothelial cells or microparticles derived from these cells [20]. Besides the cascade model of blood coagulation, a cell-based model of coagulation has been proposed too [18]. According to this model, hemostasis in vivo occurs in three overlapping phases: initiation of coagulation on TF-bearing cells; amplification of the coagulant response on the surface of activated platelets; and propagation, in which hemostatic amounts of thrombin are generated on the platelet surface [18].

The coagulation cascade is a tightly regulated process with many positive but also negative feedback loops. Tissue factor pathway inhibitor (TFPI) inhibits the initiation of blood coagulation at the level of TF/FVIIa. Antithrombin inactivates almost all coagulation enzymes. Activated protein C (APC) inactivates cofactors FVIIIa and FVa thereby dampening the prothrombinase and tenase complexes. Protein S, a cofactor of protein C, also has an APC independent anticoagulant effect by direct inhibition of the formation of prothrombinase and tenase complexes on a phospholipid surface. The most recently described component of the anticoagulant system is the protein Z/Z-dependent protease inhibitor system that inhibits FXa, FXIa and FIXa by different mechanisms [21].

Fibrinolysis is the physiological mechanism that dissolves the fibrin clot in order to restore the blood flow. The key enzyme in this process is plasmin, which is formed by the activation of plasminogen in the presence of its activators (tissue type-plasminogen-activator, t-PA and urinary-type plasminogen activator, u-PA). Plasmin cleaves fibrin into small soluble fragments. Fibrinolysis is inhibited by plasminogen activator inhibitors (PAI-1 and PAI-2) as well as by plasmin inhibitors (α_2-antiplasmin, α_2-macroglobulin, α_1-antitrypsin) [22]. TAFI, which is activated only by high thrombin concentrations, inhibits fibrinolysis through inhibition of the binding of plasminogen to fibrin [17]. FXIIIa stabilizes the fibrin clot by the formation of cross-links between the various fibrin chains.

ANNEXIN A5

Localization and exocytosis of Annexin A5

Annexin A5 (ANXA5), also known as placental protein 4 [23], vascular anticoagulant protein α [24]), calphobindin I [25], endonexin II [26], placental anticoagulant protein I [27], lipocortin V [28] and 35 γ-calcimedin [29] is the most abundant protein of the annexin family [1]. The history of ANXA5 started about 36 years ago with its isolation from placental tissues by *Bohn and colleagues* [30]. Thereafter, ANXA5 was isolated from different tissues: arteries of the umbilical cord, bovine aorta, human placenta, human and bovine lung, human and rat liver, rat heart and kidney as well as from skin, heart, spleen, uterus, skeletal muscle of a human source [24;27;31-34].

The protein is widely distributed in human tissues. ANXA5 is especially highly expressed by cells that serve a barrier function, including placental trophoblasts, vascular endothelium and endothelial cells of the choroid plexus of the brain [35-38]. The placenta is the richest source of ANXA5 in humans, where ANXA5 is expressed by trophoblasts, a layer of epithelial cells covering all villous trees and separating the villous interior from the maternal blood [39]. The human placental trophoblasts have a two-layered structure consisting of the underlying layer of progenitor mononucleated cytotrophoblasts and the outer layer of syncytiotrophoblasts formed by fusion of cytotrophoblasts. ANXA5 localizes within the cytosol of placental syncytio- and cytotrophoblasts [38] and is also present on the apical membranes of syncytiotrophoblasts, where it is bound to the anionic phospholipid phosphatidylserine (PS) externalized during trophoblast differentiation and fusion [36;40;41]. It has been suggested that ANXA5 could serve as a local antithrombotic protein in the maintenance of blood fluidity in the placenta [42;43]. Furthermore, ANXA5 is present in large amounts in cultured human umbilical vein endothelial cells (HUVEC; 3.6×10^{-12} g/cell) (35). ANXA5 is also found inside blood cells: leukocytes (2.8×10^{-14} g/cell), platelets (4.5×10^{-16} g/cell) and erythrocytes (2.3×10^{-16} g/cell) [35]. However, stimulation of platelets with strong agonists (e.g., thrombin, ADP, collagen) does not result in release of ANXA5 from platelets, suggesting that ANXA5 could not function as a natural systemic anticoagulant [35]. ANXA5 is also strongly expressed in tissues involved in secretory processes (prostate, testis and the islets of Langerhans) [37]. In pathological specimens, the abundant presence of ANXA5 has been found in atherosclerotic lesions of the coronary arteries [37;44].

As other annexins, ANXA5 is mainly an intracellular protein because it lacks a signal peptide required for the classical endoplasmic reticulum - Golgi secretion pathway. In mammals, there are more than 20 polypeptides (e.g., fibroblast growth factor, interleukin 1β, platelet-derived growth factor etc.), which lack the signal peptide [45;46]. Nevertheless, proteins lacking the typical signal peptide could reach the extracellular environment through onclassical secretion pathways (i.e., independent of the endoplasmic

reticulum and Golgi apparatus). Recent evidence indicates that members of the annexin family can also be released into the extracellular environment by nonclassical exocytosis pathways. Annexin A1 is actively secreted into human seminal plasma [47], and the glucocorticoid-induced externalization of Annexin A1 from folliculo-stellate cells has been proposed to be mediated by the ATP-binding cassette transporters [48]. Annexin A2 translocates from the cytoplasm to the cell surface in response to a brief period of temperature stress both *in vitro* and *in vivo* [46]. Up to now, the mechanisms by which ANXA5 is externalized from cells are still unknown. It is known that ANXA5 could be released nonspecifically from injured or dying cells [49;50]. ANXA5 has been found in extracellular fluids like blood plasma, cerebrospinal fluid, amniotic fluid and urine [35;49-52]. However, ANXA5 levels present in extracellular fluids are considerably lower compared to its intracellular levels. Plasma levels of ANXA5 measured in EDTA plasma of healthy controls are in the range 0 - 5 ng/ml [35].

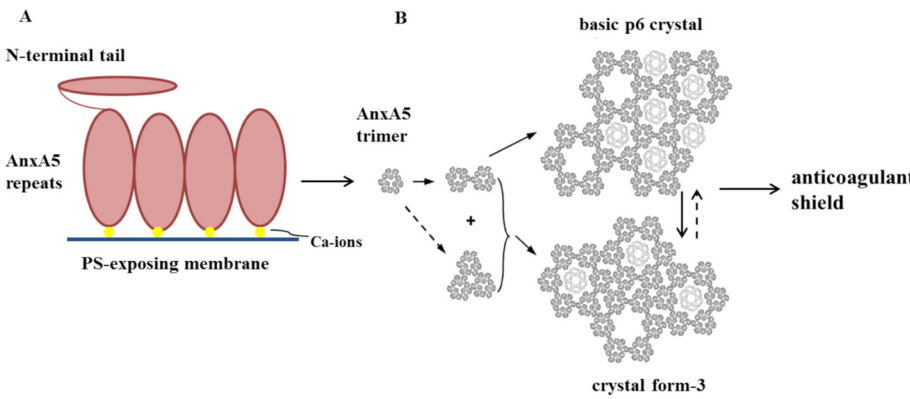

Figure 2. A model of the Annexin A5 2D crystallization. A, A schematic representation of the Annexin A5 molecule bound to a negatively charged phospholipid membrane in the presence of Ca2+-ions. B, Upon binding to a PS-exposing surface, monomeric Annexin A5 forms trimers, which associate into 2D crystal forms. Adapted from *Oling et al.*, 2001 [63]

Annexin A5: structure and phospholipid-binding properties

ANXA5 is a non-glycosylated single chain protein of 320 amino acids with a molecular weight of 35.7 kDa. The primary structure of its polypeptide is organized in a short N-terminal tail and a conserved C-terminal core, which comprises four repeats (**Figure 2**). The repeats are homologous sequences of approximately 65-70 amino acids, and each repeat contains the area known as the 'endonexin loop' (~17 amino acids long) for the calcium binding sites [53]. The tertiary structure of an ANXA5 molecule was resolved by *Huber and colleagues* in 1990 [54]. X-ray crystallography revealed that the polypeptide chain of ANXA5 is folded into four domains. Each domain comprises five alpha-helices wound into a right-handed superhelix. The domains are tightly associated to form two modules (domains I, IV) and

(domains II, III). The interaction between domain I and IV is mediated in a non-covalent manner by the amino-terminal tail. Domain II and III are linked via a short interhelical turn. The ANXA5 molecule is slightly bent, with both a convex and a concave face [54;55]. A short amino-terminal tail is located at the concave surface, and the calcium- and phospholipid-binding sites - on the convex, membrane-facing side of the ANXA5 molecule. The Ca^{2+}-binding sites are suited as protruding loops; three of them are located in homologous conserved segments (Met/Leu)-Lys-Gly-(Ala/Leu)-Gly-Thr within the "endonexin loop" in domains I, II, IV [56;57]. In the presence of a high Ca^{2+} concentration (10-60 mM), structural changes in domain III lead to the formation of a new calcium site. *Sopkova et al.* showed that the formation of this new calcium site causes the displacement of a Tryptophan residue at position 187 from a buried to an exposed conformation on the one hand and results in the loss of two calcium sites in domains II and IV on the other hand [58].

ANXA5 binds in a Ca^{2+}-dependent manner to phospholipids, with a preference for negatively charged phospholipids such as phosphatidylserine (PS) [59]. Ellipsometric experiments showed that half-maximal binding of ANXA5 to phospholipid bilayers consisting of 5 mol% PS/95 mol% Phosphatidylcholine (PC) and 20 mol% PS/80 mol% PC is reached at the millimolar Ca2+ levels of 1.5 mmol/L and 0.22 mmol/L, respectively [59]. ANXA5 binds with high affinity to a 20% PS/80% PC surface (dissociation constant [Kd] <1-2 x 10^{-10} mol/L) [59;60]. Besides its binding to model membranes, ANXA5 also binds to PS-exposing cell membranes of stimulated, apoptotic and necrotic cells [61;62]. Based on its capacity to bind PS-exposing membranes with high affinity, ANXA5 is widely used for visualization of cell death, both *in vitro* and *in vivo*, in animal models and in patients [64]. Furthermore, the ANXA5 affinity assay, which comprises the incubation of cells with labelled ANXA5 and the vital dye propidium iodide, allows us to discriminate between living cells and cells with the apoptotic or necrotic phenotypes [62].

ANXA5 molecules exist in two forms: a water-soluble and membrane-bound form. Cryo-electron microscopy and X-ray crystallography studies demonstrated that the structure of water-soluble ANXA5 is almost identical to that of its membrane-bound form [57;65;66]. This means that upon binding to a phospholipid membrane, ANXA5 maintains its convex-concave shape, does not become flat and does not undergo significant structural changes [6]). This also indicates that Ca^{2+}-binding loops are located at the different distances from the membrane surface. In this context, domain III is the most distinct from the membrane surface and domain II is the closest as shown by X-ray crystallography and atomic force microscopy [66;67].

In solution, ANXA5 molecules are monomers and do not assemble into crystal lattices. At phospholipid surfaces containing negatively charged phospholipids (e.g., planar phospholipid monolayers, vesicles, phospholipid bilayers), ANXA5 self-assembles into two-dimensional (2D) ordered crystal structures [63;65;67;68]. *Brisson and colleagues* showed by electron

microscopy and atomic force microscopy that upon binding to PS, ANXA5 forms trimers, which are the basic building blocks of a 2D crystal lattice, and these trimers associate via protein-protein interactions into four 2D crystal forms (basic P6 crystal; crystal form-1, -2 and -3) [63] (**Figure 2**). The type of a 2D crystal depends on PS content and Ca^{2+} concentration. The low-density honeycomb-like P6 crystals, which are commonly found, are formed at low-to-medium PS content (5 to 20%). The high-density P3 crystals are found at high PS content (above 40%) and could also be formed from the P6 form as a result of a crystal phase transition [63;69]. It should be noted that the basic ANXA5 P6 crystals are open lattices with the presence of large protein-free spaces (~ 9 nm in diameter) around the six-fold symmetry centres [63;70]. It has been suggested that other annexins or proteins could be integrated within the ANXA5 2D arrays and that such assemblies could be of physiological importance [70-72], for example, in cell membrane repair [73]. It has recently been demonstrated that ANXA5 also trimerizes at the surface of apoptotic Jurkat cells [74]. *Ungethüm and colleagues* were the first to show by using a FRET (Fluorescence Resonance Energy Transfer) approach combined with transmission electron microscopy that ANXA5 forms trimers and these trimers assembles into 2D ordered crystals on a cellular membrane [74]. Not only ANXA5 but also other annexins form 2D crystals of the trimers on the surface of artificial membranes. Trimerization has been observed for ANXA4 [75], ANXA6 [76;77] and ANXB12 (from hydra) [71].

Annexin A5 biological activities

ANXA5 exhibits a variety of biological activities inside the cell and in the extracellular environment as well.

Annexin A5 as an anticoagulant and antithrombotic protein

The first observed *in vitro* activity of ANXA5 was its anticoagulant activity [24;31]. ANXA5 is supposed to belong to the physiological anticoagulants. As mentioned above, the physiological anticoagulant system includes inhibitors of the serine proteases (antithrombin, heparin cofactor II, α_2-antiplasmin, α_2-macroglobulin, α_1-antitrypsin), inhibitor of TF (TFPI) and inhibitors of the nonenzymatic cofactors FVa/FVIIIa (protein C/protein S system in combination with either Thrombomodulin or endothelial protein C receptors (EPCRs)). The mechanism of ANXA5-based anticoagulation differs from that of other known physiological anticoagulants. It has been shown that ANXA5 interferes in the complicated process of blood coagulation through nonenzymatic interactions with negatively charged phospholipids and does not anticoagulate through proteolysis or direct/indirect inhibition of enzymes [31;78]. That means that ANXA5 interference occurs indirectly and thus, there is no direct interaction between ANXA5 and coagulation factors. Data from fluorescence correlation spectroscopy experiments on supported artificial membranes showed that ANXA5 and FVIIIa do not interact directly [79]. ANXA5 anticoagulant activity is based on its high affinity binding to anionic phospholipids, especially PS (dissociation constant [Kd] less than 0.2 nM) [59] and reducing the availability of PS for other ligands by shielding PS lipids upon binding [78;79]. ANXA5 competes with

coagulation factors for the same phospholipid-binding sites on the plasma membrane and displaces coagulation factors from procoagulant surfaces, thereby impairing their complex formation.

ANXA5 is widely known as a strong inhibitor of blood coagulation [27;31;80;81]. *In vitro* studies have shown that ANXA5 inhibits the assembly of coagulation enzyme-substrate complexes on artificial and cellular membranes [31;61;78]. In a human *ex vivo* thrombosis model, in which nonanticoagulated blood was perfused over the extracellular matrix of tumor necrosis factor-stimulated endothelial cells, ANXA5 has been shown to prevent thrombus formation [82]. Under low shear rates conditions reflecting blood flow in small veins, ANXA5 inhibited both platelet adhesion and fibrin deposition. At higher shear rates (*i.e.*, comparable with those in medium-sized arteries), ANXA5 impaired platelet aggregation but had no inhibitory effects on platelet adhesion [82]. *In vitro* perfusion experiments demonstrated that the ANXA5 inhibitory potential on platelet thrombus formation was comparable with that of a direct thrombin inhibitor, hirudin, or an antibody against TF [83]. ANXA5 interferes in the coagulation cascade at a triple level – inhibiting prothrombinase, factor Xase and also interfering with the action of FVIIa-TF complex [83]. It has been proposed that it could be the explanation for the powerful inhibitory effects of ANXA5 [83].

In *in vivo* animal models, ANXA5 is an effective inhibitor of thrombus formation in a laser- and photochemically-induced venous thrombus model in rats [84]. Furthermore, ANXA5 accumulates at the site of injured jugular veins in rabbits and decreases fibrin deposition [85]. ANXA5 is also an effective inhibitor of arterial thrombosis in a rabbit carotid artery injury model by reducing thrombus weight in a dose-dependent manner, inhibiting fibrin deposition and reducing platelet accumulation at the site of arterial injury [86]. More importantly, in an arterial thrombus model, the inhibitory effects of ANXA5 were not associated with bleeding tendency, even at supraclinical ANXA5 concentrations (*i.e.*, greater than that required to induce an antithrombotic effect), whereas the therapy with heparin induced bleeding at extravascular sites [86].

Recombinant fusion proteins that have been constructed based on the structure of the native ANXA5 molecule exhibit stronger anticoagulant and antithrombotic activities both *in vitro* and *in vivo* compared to those of the native ANXA5. An example of these proteins is a two-domain recombinant fusion protein consisting of a human ANXA5 domain and a Kunitz protease inhibitor domain (i.e., serine protease inhibitor) [13;87]. These fusion proteins bind to PS-exposing membranes (sites of thrombogenesis) via the ANXA5 domain that greatly facilitates the binding of the Kunitz protease inhibitor domain to the active site of the membrane-associated enzyme/cofactor assemblies. It has been demonstrated that fusion proteins are powerful inhibitors of coagulation reactions in an *in vitro* TF-initiated clotting assay and they have higher antithrombotic activities in a mouse arterial thrombosis model *in vivo* compared to those of the native ANXA5 [13].

One of these fusion proteins, ANXA5-6L15 (an aprotinin mutant) efficiently inhibits the TF-FVIIa complex on the PS/PE-exposing membranes in an *in vitro* clotting assay and has potent cardioprotective features in a rat cardiac ischemia-reperfusion injury model [87]. More importantly, ANXA5-6L15 does not induce a bleeding tendency and is more effective as a cardiprotector than a direct thrombin inhibitor, hirudin. It has been suggested that ANXA5-6L15 could be a promising drug candidate for cardioprotection against myocardial ischemia-reperfusion injury [87].

Another example of fusion proteins with potent antithrombotic properties is Diannexin A5, a homodimer of ANXA5 [88;89]. The designed 14 amino acid peptide that links the monomer ANXA5 molecules in Diannexin allows the dimer to bind PS with ~10-fold higher affinity compared to the native ANXA5 monomer (Kd 0.6 nM versus 5.1 nM) [89]. In comparison with the native ANXA5, Diannexin A5 is more effective in inhibition of platelet-fibrin clot formation both *in vitro* and in a mouse laser-induced arterial thrombosis model in vivo [89]. Diannexin A5 is also highly efficient in inhibition of venous thrombosis in a rat venous thrombosis model [88].

It is generally assumed that the anticoagulant mechanism of ANXA5 could be explained by displacement of coagulation factors from procoagulant surfaces and thus, inhibiting their complex formation [78;90]. However, the mechanism of ANXA5-based anticoagulation is more complex than just the displacement concept. In 1992, *Andree and colleagues* demonstrated incomplete displacement of FXa, FVa and prothrombin from the phospholipid surface by ANXA5 [78]. It has been proposed that the formation of rigid clusters of ANXA5 may explain its anticoagulant effect and that ANXA5 arrays do not completely block binding of coagulation factors but reduce the lateral mobility of membrane-bound coagulation factors preventing thereby their complex formation [78]. The complexity of the ANXA5 anticoagulant mechanism on a cellular surface has further been supported by data of Ravassa et al., who showed that ANXA5 that self-assembles into 2D arrays at the cell membrane down-regulates the expression of the procoagulant tissue factor by inducing its internalization [91]. To date, there is no experimental data regarding the effect of ANXA5 lattice formation on the lateral mobility and complex formation of coagulation factors. Thus, the mechanism of ANXA5-based anticoagulation is still not completely understood.

Annexin A5 anti-inflammatory-related activities

ANXA5 possesses anti-inflammatory-related activities, such as inhibition of phospholipase A2 activity, inhibition of pro-inflammatory effects of oxidized low-density lipoproteins (LDLs) and regulation of interferon-gamma (IFN-γ) receptor signaling.

The activity of ANXA5 as an inhibitor of phospholipase A2 is also based on its property to shield negatively charged phospholipids, substrates for phospholipase A2. Phospholipase A2 hydrolyses membrane phospholipids releasing a fatty acid and a lysophospholipid. The free fatty acid in the form of arachidonic acid is subsequently converted into prostaglandins and leukotrienes, which are pro-inflammatory mediators [92]. Studies with artificial membranes and HL-60 cells have demonstrated that ANXA5 inhibits the phospholipase A2-mediated hydrolysis of membrane phospholipids in a calcium-dependent manner [93-95] and that mutations in the Ca^{2+}-binding sites of ANXA5 could modify its inhibitory effect [94]. However, inhibition of cytosolic phospholipase A2 activity by ANXA5 was shown to be much smaller and less specific than that by ANXA1 [96]. Thus, ANXA1 but not ANXA5 is thought to be an endogenous negative regulator of cytosolic phospholipase A2 [96].

The next property of ANXA5, which could contribute to its anti-inflammatory effects, is an association of ANXA5 with LDL isolated from human plasma. It has been reported that ANXA5 binds specifically to oxidized phosphatidylcholine present in oxidized LDL but not to native LDL [97]. Oxidized phospholipids generated during oxidative modification of LDL possess a variety of atherogenic and pro-inflammatory effects [97;98]. A recent report demonstrated inhibitory capacities of ANXA5 on pro-inflammatory effects of oxidized LDL and lysophosphatidylcholine in vitro and in a mouse intra-peritoneal recruitment model *in vivo* [99]. Whether ANXA5 influences binding and uptake of oxidized LDL by macrophages is not completely understood. One study reported that ANXA5 binding to oxidized LDL did not influence binding of oxidized LDL to monocytic U937 cells and subsequently, had no effect on LDL uptake by these cells [97]. In a recent study, inhibitory effects of ANXA5 on oxidized LDL binding and uptake by macrophages have been reported [99]. It should be noted that enhanced uptake of oxidized phospholipids by macrophages might promote the formation of foam cells, which play a key role in atherogenesis, and therefore, accelerate atherosclerosis [97;99]. Whether ANXA5 really inhibits uptake of oxidized LDL by macrophages deserves further investigation.

ANXA5 is also able to interact with the intracellular domain of the R2 subunit of the human interferon-gamma (IFN-γ) receptor to form a stable complex and to negatively regulate IFN-γ signalling [100].

Annexin A5 anti-apoptotic activities

Cells undergoing apoptosis (programmed cell death) and necrotic cells are characterized by externalization of PS, one of the "eat me" signals for phagocytosis by macrophages. ANXA5, which binds with high affinity to PS, influences membrane dynamics of the PS-expressing cell surfaces. ANXA5 inhibits phagocytosis of the apoptotic Jurkat cells by J774 macrophages and that inhibition of phagocytosis requires high ANXA5 concentrations (about 20 μg/ml or 560 nM) [101]. The inhibitory action of ANXA5 is based not only on PS shielding but also on the internalization of receptors/ligands that are

in the proximity of PS (101). Besides its inhibitory effects on phagocytosis, ANXA5 has been reported to influence the apoptotic cell death program after binding to CEM human T-lymphoma cells by delaying the activation of caspase-3 processing [102]. On the other hand, ANXA5 binding to Fas-stimulated human Jurkat T-lymphoma cells shows no effect on progression of apoptosis [103].

ANXA5 has also been proposed to be an important modulator of the immune response against tumor cells as well as that against dying and dead cells in mouse models *in vivo* by inhibiting cell uptake by macrophages [104;105]. *Munoz et al*. showed that necrotic human T cells introduced into the circulation of wild-type mice cause a strong delayed-type hypersensitivity reaction (DHT; i.e., the reaction that characterized by an induction of a specific T cell response), whereas ANXA5 deficient mice (i.e., lacking endogenous ANXA5) showed almost no DHT reaction after immunization with necrotic cells [104].

Other Annexin A5 activities

In the intracellular environment, ANXA5 has been suggested to be **involved in signal transduction pathways**. As an example, ANXA5 inhibits the activity of protein kinase C isoforms with a Ca^{2+}/phospholipid-binding C2 domain [106;107]. The data from in vitro studies indicate that ANXA5 inhibits the activity of protein kinase C via a mechanism of phospholipid sequestration and the Ca^{2+}-binding site located in domain 1 of ANXA5 plays a crucial role in protein kinase C inhibition [107]. It has also been shown that ANXA5 could function as a signalling protein for vascular endothelial growth factor receptor by directly interacting with the intracellular domain of the receptor and could be involved in the regulation of vascular endothelial cell proliferation [108].

ANXA5 as well as ANXA6 and ANXA7 are able to form voltage-dependent calcium channels within artificial lipid bilayers [55;109]. Two main models were proposed to explain **the ion channel activity of ANXA5**: one involves a hydrophilic pore, which lies at the centre of the ANXA5 molecule and forms a prominent ion channel; the second model involves the penetration of ANXA5 into the membrane [55]. Data of *Isas and colleagues* support the hypothesis that ANXA5 could insert into the lipid bilayer at mild acidic conditions (pH 5-6) [110]. However, it is still not clear if there is any relevance of the ion channel activity of ANXA5.

Recently, a few new activities of ANXA5 were described. *Brisson and colleagues* reported that ANXA5, a protein that self-assembles into two dimensional (2D) arrays on the cell membrane, **promotes membrane repair** [73]. ANXA5 molecules bind to PS exposed at ruptured membrane edges and form 2D arrays, which stabilize the membrane, prevent wound expansion and promote membrane resealing. In contrast, an ANXA5 mutant that lacks the ability of forming 2D arrays and ANXA5-null mouse perivascular cells

were unable to promote membrane repair, showing a severe membrane defect. In this context, the capacity of ANXA5 to promote membrane repair is of vital importance for cells as failure to reseal membranes leads to cell death or may contribute to the development of degenerative diseases. It has been suggested that ANXA5 may be of therapeutic value in pathologies associated with defects in the membrane repair machinery [73].

Another ANXA5 activity is based on its **binding to intact lipopolysaccharide (LPS)** expressed in the outer membrane of Gram-negative bacteria and **to the lipid A domain of LPS**, which is mainly responsible for the endotoxin effect of LPS [111]. It has been shown that ANXA5 inhibits LPS-induced production of proinflammatory cytokines such as TNF-α *in vitro* and in mice *in vivo*. Therefore, it has been proposed that ANXA5 could play a role in host defense against Gram-negative endotoxemia by reducing LPS effects *in vivo* and may protect against attack induced by LPS [111].

ANNEXINOPATHIES

General overview of annexinopathies

The idea that annexins and ANXA5 particularly could be of biological importance is based on their wide distribution in tissues and their membrane-related biological activities. Although the gene knock-out models at the cellular and the animal levels have provided some evidence for the possible biological roles of annexins, the precise function of the annexin family as a whole and that of individual annexins still remains to be proven. It has been shown that the ANXA1 knock-out mouse has changes in the inflammatory response, supporting thereby the role of ANXA1 in inflammation [112]. Examination of the ANXA2 knock-out mice demonstrated the involvement of ANXA2 in the regulation of fibrinolysis and neovascularization [113]. Mice lacking ANXA5 are viable, fertile and do not show a thrombotic phenotype [114]. Later, it has been demonstrated that loss of maternal ANXA5 is associated with reduction in litter size and foetal weight as well as with placental thrombosis, providing thereby evidence that the maternal ANXA5 acts as an antithrombotic protein [115]. In the case of the ANXA5 knock-out mouse model or in some other cases, it is important to keep in mind that changes in expression levels of one individual annexin could affect the expression of other members of the annexin family and that the deficiency of one could lead to compensatory upregulation of another [114;116;117]. It has also been proposed that similar changes in expression levels of annexins can occur in disease states in humans [117].

The term "annexinopathy" introduced by *Jacob H. Rand* in 1999 links abnormalities in annexin expression levels, which could be of genetic or acquired origin, to disease conditions [4]. The ANXA5-related annexinopathy, one of the first described annexinopathies, is associated with the antiphospholipid syndrome (APS). The APS manifests as vascular thrombosis and/or recurrent pregnancy loss in patients with the persistent

presence of antiphospholipid antibodies (APLs) in plasma. A decreased ANXA5 expression has been reported on placental villi of women with APS [118] as well as on cultured placental trophoblasts and endothelial cells exposed to IgG fractions from APS patients [6]. APL-mediated reduced expression of ANXA5 on placental syncytiotrophoblasts and on endothelial cells was shown to explain recurrent pregnancy loss and vascular thrombosis in APS [6]. The deficiency of ANXA5 on cell surfaces is associated with acceleration of plasma coagulation as well as with an increased procoagulant activity of cultured trophoblasts/endothelial cells *in vitro* [6;119] and placental thrombosis in an animal model *in vivo* [43]. One of the mechanisms responsible for APL-mediated acceleration of blood coagulation is the disruption of the protective antithrombotic shield of ANXA5 on the cell surfaces by APLs. Evidence for that came from atomic force microscopy experiments showing varying degrees of disruption of the ANXA5 2D ordered crystallization on the phospholipid bilayers in the presence of monoclonal human APL and ß2-glycoprotein I (the major APL co-factor) [120]. It is known that APLs are a heterogeneous population of auto-antibodies. In this respect, another human monoclonal antibody (i.e., CIC15) was unable to disrupt the 2D ordered arrays of ANXA5 [70].

The list of annexinopathies is still not complete. Modern genetic approaches allow us to identify new mutations and/or polymorphic variations within the annexin genes, which through dysregulation of annexin expression levels could contribute to disease states. We believe that future research will reveal a new class of annexin genetic disorders, which in turn may lead to unravelling of the exact biological functions of annexins.

***Annexin A5* gene; *Annexin A5* genetic variations in disease states**

The gene encoding ANXA5 protein consists of an untranslated exon 1 and 12 coding exons that span approximately 29 kb on the human chromosome 4q27 [121-123] (**Figure 3**). The human *ANXA5* promoter lacks a TATA box and contains three transcription start sites (tsp) [122]. Basal transcription has been shown to be controlled by the two SP1 (specificity protein 1 transcription factor) sites, which are located in the CpG-island with an average 75% G+C nucleotide content [123]. The SP1 transcription factor binds directly to the GC-rich motifs of DNA and enhances gene transcription. The human *ANXA5* gene also contains an upstream endogenous retroviral long terminal repeat (437 bp), which has impact on the regulation of *ANXA5* transcription. The region downstream of the tsp1 in exon 1 (from +31 to +79) was found to be absolutely essential for the *ANXA5* transcriptional activity [123]. In this segment (called an alternative promoter), two main elements were identified: DNA motif A (*GCGTTT*) with a binding site for c-MYB (transcriptional activator Myb) and DNA motif B (*GCAGCTGC*) containing an AP4 (activating enhancer-binding protein 4) site. The MED-1 element (Multiple start site Element Downstream) (*GCTCCG*), which is an extension of the motif B, has been proposed to be relevant in gene transcriptional regulation. However, its role in transcriptional control needs to be clarified [123].

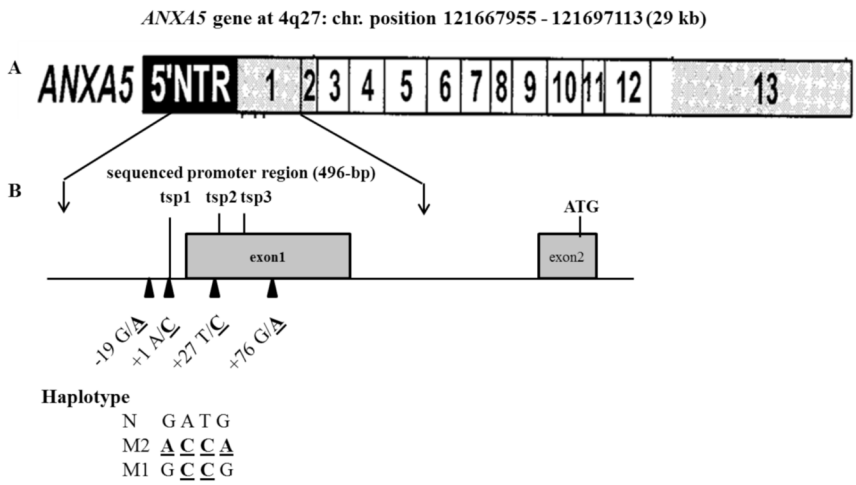

Figure 3. The human *Annexin A5* gene and *Annexin A5* promoter variations. A, A schematic representation of the *Annexin A5* gene displays the upstream non-transcribed region (NTR) and 13 exons. The untranslated exons are depicted in grey, coding exons – in white. The promoter region is in black. Adapted from *Carcedo et al.*, 2001 (123). B, Polymorphic variations in the 496-bp promoter region of the *Annexin A5* gene and three common promoter haplotypes, according to *Bogdanova et al.*, 2007 (126). Nucleotide numbering is from the first transcription start point (tsp1). Minor alleles are in bold and underlined. The ATG codon means the translation initiation codon.

It is known that gene expression is regulated at various levels, including 1) transcription, 2) regulation at the level of RNA (processing, transport, translation and degradation of the mature mRNAs) and 3) protein processing and degradation. It is generally assumed that functional genetic variations could influence gene expression. A decreased or an increased mRNA or protein expression as a consequence of an affected gene expression might be associated with some disease features. If *ANXA5* variations affect ANXA5 expression within the vascular wall or in placental trophoblasts where large amounts of ANXA5 are present, it is expected to be associated with some clinical outcomes, such as an increased or decreased risk of placental or vascular thrombosis.

To date, our knowledge about the regulation of *ANXA5* gene expression is insufficient. In 2002, *Gonzales-Conejero et al*. showed for the first time that the single nucleotide polymorphism (SNP) rs1131239, which is located one nucleotide upstream of the ATG (AUG in the mRNA) initiation codon in exon 2 of the *ANXA5* gene, affects *ANXA5* translation efficiency resulting in higher plasma ANXA5 levels in minor rs1131239T-allele carriers [124]. The authors suggested a protective role of increased plasma ANXA5 levels in the occurrence of arterial thrombosis as the presence of the minor rs1131239T-allele was associated with a decreased risk of myocardial infarction [124]

and a lower risk of developing new thrombotic events during 36 months follow-up [125]. The above mentioned SNP rs1131239 is located in the Kozak consensus sequence (i.e. GCCRCCaugG (R = purine)), which plays a crucial role in the initiation of translation in humans [127;128]. Mutagenesis experiments showed that some nucleotides within the Kozak motif are more important than the others [128]. The nucleotides A or G at position -3 (i.e. upstream from the AUG codon) as well as G at positions +4 (i.e. downstream from the AUG codon) and -6 appeared to have the biggest contribution to the translation process whereas the contribution of the nucleotides CC at positions -1 and -2 was shown to be small (128;129). It has also been shown that only in the absence of -3R (i.e., A or G) and +4G mutations at positions -1 and -2 do exert some influence on translation [129]. Subsequent studies have confirmed the Kozak's experimental data demonstrating the lack of association between SNP rs1131239 and plasma ANXA5 levels in healthy persons [130;131]. Several research groups have investigated the clinical relevance of SNP rs1131239. The minor rs1131239T-allele was not associated with a decreased risk of myocardial infarction in the Dutch and Finnish male patients [132;133], nor was it associated with ischemic stroke [134], preeclampsia [135] and vascular thrombosis in the antiphospholipid syndrome [136].

In 2007, *Bogdanova and colleagues* reported four *ANXA5* promoter polymorphisms [126] (Figure 3). SNP rs112782763 (g. -19G>A, upstream from tsp 1), which is located in the gGCCc sequence, affects the zinc finger binding of the MTF-1 (metal-regulatory) transcription factor [137]. Substitution at rs28717001 (g. +1A>C) changes the tsp1 itself. The third SNP rs28651243 (g. +27T>C, downstream from the tsp1) disrupts a SP1 consensus sequences. Finally, SNP rs113588187 (g. +76G>A, downstream from the tsp1) destroys a BamHI restriction site in close proximity of an AP-4 (motif B)/MED-1 consensus, which in turn is essential for the full *ANXA5* promoter activity [123;126]. *Bogdanova and colleagues* also showed that the expression of the *ANXA5* gene could be influenced by two *ANXA5* promoter haplotypes [126]. The *ANXA5* promoter activity (luciferase reporter gene assay) was reduced in the M1 haplotype comprising minor alleles of two SNPs (rs28717001, rs28651243) and in the M2 haplotype consisting of four SNPs (rs112782763, rs28717001, rs28651243, rs113588187) to 57-62% and 37-42% activity of the wild-type haplotype (100%), respectively [126]. Later, the M2 *ANXA5* allele was linked to reduced ANXA5 mRNA levels in placental tissues compared to the wild-type allele [138].

The idea that the hypofunctional *ANXA5* M2 allele may promote a procoagulant state in placentas has initially been proposed by Bogdanova and colleagues. They reported that the presence of this haplotype is associated with a 2.4-fold increased risk of pregnancy loss in German females [126]. An association of the *ANXA5* M2 haplotype with recurrent pregnancy loss was further confirmed in Italian [139], Japanese [140] and Bulgarian women [141], but not in subjects from East China [142] and also not in a second cohort of Japanese women [143]. The presence of the M2 haplotype is also related to the risk factor of recurrent pregnancy loss in

polycystic ovarian syndrome [144]. Recently, it has been proposed that in patients with obstetric APS, a reduced ANXA5 expression at the placental villi through carriage of the *ANXA5* M2 haplotype may be an explanation for pregnancy loss as well as a predisposing factor for the development of APLs (so-called "genetic" revised model of APS) [145], though data confirming this idea are still not available.

An association of the *ANXA5* M2 haplotype with deep venous thrombosis (DVT) was also assessed. However, the contribution of the M2 haplotype to thrombotic risk remains debatable. In two Italian studies, it was shown that the presence of the M2 haplotype is associated with an increased risk of DVT in pregnancy/postpartum period as well as in the general population [146;147]. On the other hand, in a Spanish study, the minor rs1131239T-allele, which is located in the *ANXA5* Kozak sequence and completely linked to SNPs rs112782763 and rs113588187 specific for the M2 haplotype [131;140], was not associated with a first episode of DVT [124].

OUTLINE OF THE THESIS

The aim of this thesis was two-fold: 1) to extend our knowledge on the role of ANXA5 two-dimensional lattices in ANXA5-based anticoagulation; 2) to study whether *ANXA5* genetic variations contribute to the pathogenesis of thrombosis- or inflammation-related diseases in order to get more insight into the biological significance of endogenous ANXA5 in humans.

Chapter 2 addresses the ANXA5 self-association on PS-exposing cell surfaces and the role of ANXA5 lattices in PS-depending coagulation reactions. The flow cytometric and microscopic Fluorescence Resonance Energy Transfer (FRET) approach was used to visualize both ANXA5 interactions and interactions between FIX and FVIII of the intrinsic pathway of blood coagulation on PS-exposing cell surfaces.

In **Chapter 3** we described the four common *ANXA5* haplotypes within the *ANXA5* gene upstream region in healthy controls. Furthermore, we demonstrated the variability of plasma ANXA5 levels regarding to *ANXA5* haplotypes.

In **Chapter 4** we investigated whether *ANXA5* variations and/or plasma ANXA5 levels exerted an influence on carotid atherosclerosis progression by evaluating carotid IMT (intima-media thickness, a marker of atherosclerosis) as well as contributed to cardiovascular disease (CVD) risk in patients with familial hypercholesterolemia (FH). To achieve this goal, two large cohorts of FH patients were investigated: the ASAP (Atorvastatin versus Simvastatin on Atherosclerosis Progression) and GIRaFH (Genetic Identification of Risk factors in Familial Hypercholesterolemia).

In **Chapter 5** we aimed to evaluate whether *ANXA5* promoter SNPs and/or common haplotypes are associated with deep venous thrombosis (DVT) in the Dutch general population. For this study, we used a case-control study on risk factors for DVT, the Amsterdam Case-control Thrombophilia (ACT) study, and a large group of population controls (Nijmegen Biomedical Study, NBS).

In **Chapter 6** we studied an association of *ANXA5* common haplotypes with thrombosis (either arterial or venous) and pregnancy morbidity in APS (antiphospholipid syndrome) patients.

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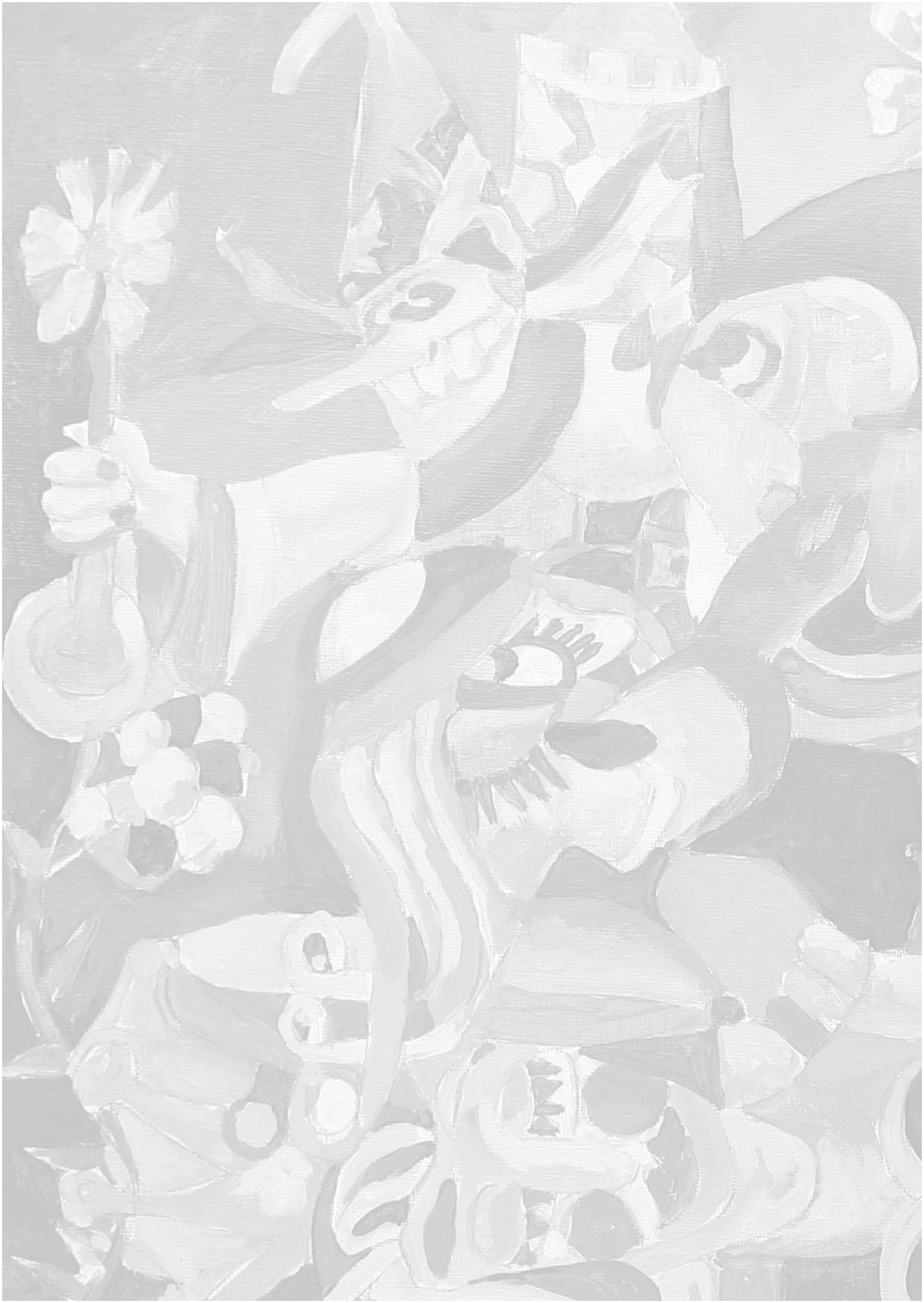

Chapter 2

Annexin A5 membrane-bound lattices interfere in tenase complex formation on phosphatidylserine-exposing cells: evidence from a Fluorescence Resonance Energy Transfer study

Larissa Hiddink, Nancy Jacobs, Rob Woestenenk, Jack A.M. Fransen, Bent Brachvogel, Arnoud Loof, Jan Boezeman, Waander L. van Heerde

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ABSTRACT

Background: Annexin A5 (ANXA5) anticoagulant activity is based on its high affinity for negatively charged phospholipids. The formation of ANXA5 two-dimensional (2D) lattices has been proposed to be relevant for ANXA5-mediated anticoagulant effects.
Objectives: To get more insight into the role of ANXA5 lattices in phosphatidylserine (PS)-depending coagulation processes on the cell surface.
Methods and Results: We used a flow cytometric and confocal Fluorescence Resonance Energy Transfer (FRET) approach to visualize FIX-FVIII interactions. Upon binding to ionomycin-activated COS1 cells, coagulation factors FIX and FVIII interact with each other forming the tenase complex (fluorescence FRET signal 49.1±10.6%). The interactions between FIX and FVIII were inhibited by pre-assembled ANXA5 at 5 nM (FRET signal 4.1±1.1%). ANXA1, which binds to PS without forming 2D lattices, reduced FIX-FVIII interactions to a lesser extent under the same conditions (FRET signal 23.1±1.0%). Higher ANXA5 concentrations were required to block the binding of FIX and FVIII to activated cells. Using the same FRET approach, we observed that ANXA5 assembles into 2D tightly-packed lattices on artificial membranes and PS-exposing cells such as ionomycin-activated platelets and COS1 cells. ANXA1 showed hardly any FRET upon binding to ionomycin-activated platelets or stimulated COS1 cells, an indicator of membrane-bound unstructured clusters.
Conclusions: Our observations suggest that ANXA5 lattice formation on PS-exposing cell surfaces contributes to ANXA5 anticoagulant properties. We assume that ANXA5 lattices inhibit tenase complex formation not only by shielding of PS but also by reducing the lateral mobility of PS-bound coagulation factors.

INTRODUCTION

Thrombosis is a leading cause of morbidity and mortality in the developed world [1,2]. Many genetic and acquired risk factors affecting the hemostatic balance could contribute to the formation of a clot within a blood vessel [2,3].

Annexin A5 (ANXA5), a Ca^{2+}-dependent phospholipid-binding protein of the annexin family, is involved in the pathogenesis of thrombotic disorders [4]. This protein possesses strong anticoagulant and antithrombotic properties, both in vitro and in vivo [5-9], which are based on its binding to anionic phospholipids exposed by apoptotic or activated cells and on shielding of thrombogenic cell surfaces from availability for coagulation reactions. The ANXA5 antithrombotic shield on endothelial cells as well as on placental trophoblasts may be disrupted by antiphospholipid antibodies that results in vascular thrombosis and recurrent pregnancy loss in the antiphospholipid syndrome [4,10]. Decreased ANXA5 placental expression through carriage of the M2/ANXA5 haplotype has recently been proposed to be a predisposing factor for thrombophilia-related obstetric complications [11]. In addition, reduced binding of ANXA5 to endothelial cells is associated with atherosclerotic changes and is a potential mechanism for atherothrombosis in systemic lupus erythematosus patients [12].

ANXA5 anticoagulant properties have been thoroughly examined. In vitro studies have shown that ANXA5 targeting the anionic phospholipids inhibits the assembly of coagulation enzyme-substrate complexes on artificial and cellular membranes: a) prothrombinase (activated factor V (FVa)+FXa); b) intrinsic tenase (FVIIIa+FIXa); c) tissue factor+FVIIa [5-7]. ANXA5 additionally blocks fusion of activated platelets with tissue factor-bearing microvesicles [13]. However, the anticoagulant mechanism of ANXA5 seems to be more complex than just displacement of coagulation factors from procoagulant membranes. It has been demonstrated that, upon binding to anionic phospholipids, ANXA5 self-assembles into two-dimensional (2D) trimer-based lattices on synthetic as well as on cellular membranes [14-16]. Andree and colleagues were the first to suggest that ANXA5 anticoagulant activity may be explained by the reduced lateral mobility of coagulation factors in rigid sheets of clustered ANXA5 molecules covering the model membrane [6]. In addition, ANXA5 appears to down-regulate the expression of the procoagulant tissue factor by inducing its internalization, supporting the complexity of the anticoagulant mechanism of ANXA5 [17].

The present study was performed in order to get more insight into the role of ANXA5 lattices on PS-exposing cell surfaces in coagulation reactions. We visualized interactions of coagulation factors of the tenase complex (i.e. FIX-FVIII interactions) as well as ANXA5-ANXA5 interactions on cell surfaces using a FRET (Fluorescence Resonance Energy Transfer) approach. FRET measures the energy transfer of an excited donor fluorophore to an

acceptor fluorophore in a dipole-dipole interaction, resulting in the quenching of the donor's fluorescence intensity and an increase (sensitization) in the acceptor's emission intensity [18,19]. This mechanism requires resonance interaction between donor and acceptor over distances of 2-10 nm and spectral overlap between the donor's emission spectrum and the acceptor's excitation spectrum. Using a flow cytometric (FCM) and confocal acceptor-sensitized emission FRET approach, we determined ANXA5 self-association on model membranes as well as on PS-exposing cells. The same approach was also applied to assess an effect of ANXA5 membrane-bound assemblies on FIX-FVIII interactions on the surface of ionomycin-activated COS1 cells.

MATERIALS AND METHODS

Preparation of recombinant human ANXA5

Recombinant human ANXA5 was prepared as described [20]. ANXA5 was purified using a DEAE (Amersham Biosciences) and Resource Q column (Pharmacia) installed at a fast protein liquid chromatography apparatus (Pharmacia). The protein appeared to be free of contaminants as determined by SDS-PAGE and Surface-Enhanced Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (SELDI-TOF MS; Bio-Rad). ANXA5 concentrations were measured using a Zymutest ANXA5 ELISA kit (Hyphen Biomed).

Labeling and separation of labeled ANXA5

ANXA5 was labeled with Alexa Fluor 488 (A488), A546 or A647 monosuccinimidyl-esters (Molecular Probes, Invitrogen) according to the manufacturer's instructions. Alexa labeled ANXA5 molecules with different stoichiometries were separated by FPLC (Pharmacia) using a Resource Q column. The dye to protein ratios were checked by SELDI-TOF MS. For that, labeled ANXA5 was spotted onto a normal phase protein chip (NP20; Bio-Rad). Sinapinic acid (Bio-Rad) prepared in 50% acetonitrile/0.1% trifluoroacetic acid was applied twice (1 µl each time and 2 minutes apart) and the chip was allowed to air-dry prior to reading on a SELDI-TOF MS. ANXA5 concentrations were determined by a Zymutest ANXA5 ELISA (Hyphen Biomed). For FCM and microscopic FRET experiments, we only used labeled ANXA5 with 1:1 stoichiometry.

Labeling of coagulation factors and ANXA1

For FCM and imaging experiments, recombinant human FIX (nonacog alfa; Wyeth Pharma S.A.) and FVIII (octocog alfa; Baxter S.A.) were labeled with Cy3 and Cy5 monoreactive sulfoindocyanine N-hydroxysuccinimidyl esters (Amersham Biosciences, Europe) following the manufacturer's instructions. The Cy3 dye to FIX labeling ratio (i.e. molecules of dye per molecule of protein) determined by absorbance (Nanodrop ND-1000 spectrophotometer, Isogen Life Science) at 280 nm and 550 nm was 2.5:1. The degree of Cy5-labeled FVIII was determined by absorbance at 280 and 650 nm and the dye/protein labeling ratio constituted 4.5:1. Recombinant mouse ANXA1, kindly provided by dr. B. Brachvogel (University of Cologne, Germany), was labeled with A546 or A647 monosuccinimidyl-esters (Molecular Probes,

Invitrogen) according to the manufacturer's protocol. The A546 and A647 dye to ANXA1 labeling ratios determined by the Nanodrop ND-1000 spectrophotometer and SELDI-TOF MS were 2.5:1 and 3.5:1, respectively.

Preparation of multilamellar lipid vesicles

Phosphatidylserine (PS), phosphatidylethanolamine (PE) and phosphatidylcholine (PC) were from Avanti Polar Lipids (USA). 20 mol% PS, 20 mol% PE and 60 mol% PC were mixed in a glass tube under nitrogen conditions, dried with nitrogen and resuspended in Tris-buffered saline (TBS: 50 mM Tris-HCl (pH 7.4), 150 mM NaCl). A phospholipid solution was vortexed to give a suspension of multilamellar lipid vesicles (MLVs). MLVs were used at 5 μM final concentration.

Preparation of quiescent and ionomycin-activated platelets

Citrate (3.8 %) venous blood from healthy volunteers was collected with a 19-gauge needle. The citrated blood was centrifuged at 150 g without brake at RT for 15 minutes. Platelet rich plasma was acidified by addition of 1/10 volume acid-citrate-dextrose buffer (ACD: 85 mM trisodium citrate, 71.4 mM citric acid • H2O, 111 mM glucose). Platelets were concentrated by an additional centrifugation step at 330 g without brake for 15 minutes. The pellet was resuspended twice in Hepes-Tyrodes buffer (HT: 145 mM NaCl, 5 mM KCl, 0.5 mM Na_2HPO_4 • 2 H_2O, 1 mM $MgSO_4$ • 7 H_2O, 10 mM HEPES, 5.5 mM glucose, pH 6.5 and 7.25) containing prostaglandin I_2 (10 ng mL^{-1}) (Cayman Chemical, USA). Washed platelets (100×10^9 L^{-1}) were activated by using 7 μM ionomycin (Sigma Aldrich) and 2.5 mM $CaCl_2$ at RT for 15 minutes. For FCM experiments, 0.5-1.0 $\times 10^6$ activated platelets mL^{-1} were incubated with labeled ANXA5 (20 nM) or ANXA1 (40 nM) in the presence of 2.5 mM $CaCl_2$ at RT for 10-15 minutes.

Cell preparation

COS1 cells (monkey kidney cells) were maintained in IMDM (Invitrogen) supplemented with 10% heat-inactivated fetal calf serum (FCS; Invitrogen) and 1% antibiotics (penicillin/streptomycin; ICN Biomedicals). For imaging experiments, cells were cultured in glass bottomed (0.17 mm) WillCo-dishes (WillCo Wells BV, the Netherlands) at 37°C for 24 hours. Before imaging, cells were washed with HEPES-buffered saline (HBS: 25 mM HEPES, pH 7.4, 135 mM NaCl, 5 mM KCl, 5 mM glucose) and stimulated with ionomycin (4 μM) in the presence of 2.5 mM $CaCl_2$. Confocal FRET experiments were performed with labeled ANXA5 or ANXA1 as well as with FIX-Cy3/FVIII-Cy5 under conditions described below. For FCM measurements, COS1 cells were trypsinized and washed in HBS followed by activation with 2 μM ionomycin at RT for 10-15 minutes. Activated COS1 cells (1.0-1.5 $\times 10^5$ cells mL^{-1}) were incubated with Cy3- and/or Cy5-labeled coagulation factors in the presence of 2.5 mM $CaCl_2$ at RT for 15 minutes. To study an effect of pre-bound ANXA5 or ANXA1 on FIX-FVIII interactions, activated cells were pretreated with unlabeled ANXA5 or ANXA1 (5-100 nM).

THP-1 monocytic cells were cultured in RPMI 1640 with glutamax-I and HEPES (Invitrogen) supplemented with 10% FCS and 1% penicillin/streptomycin. To obtain nucleated cells that express anionic phospholipids, THP-1 cells were treated with 5 μM ionomycin at 37°C for 20 hours. A necrotic phenotype of THP-1 cells was obtained by performing twice a freeze-thawing procedure. FCM FRET experiments were performed with 1.5-2.0 × 105 cells mL^{-1} cells incubated with labeled ANXA5 (60 nM) and 2.5 mM $CaCl_2$. 7-Amino-actinomycin D (7-AAD; Sigma Aldrich) (1μg mL^{-1}) was used to check the apoptotic and necrotic phenotype of the THP-1 cells.

Flow cytometric FRET measurements

To study ANXA5-ANXA5 as well as FIX-FVIII interactions on physiological cell membranes, we performed FRET experiments using an Epics Elite ESP (Beckman Coulter) equipped with a 488 nm Argon laser, a 532 nm solid-state laser and a 633 nm HeNe laser. ANXA5-A546 or FIX-Cy3 (donor) was excited with the 532 nm laser and the emitted signal was measured in the FL2 channel with a 575/29 nm band pass (BP) filter. ANXA5-A647 or FVIII-Cy5 (acceptor) was excited with the 633 nm laser and emission was sampled in the FL3 channel with a 675/45 nm BP filter and a 625 DLP filter. A FRET signal was obtained by excitation of the donor with the 532 nm laser and the emitted signal of the acceptor was used as readout. An average FRET efficiency was calculated by quenching of the donor [21]. The energy transfer efficiencies of FIX-FVIII interactions were corrected for the labeling ratios of donor and acceptor as previously described [21,22]. All equations are depicted in Supplemental Materials. Control experiments were performed with labeled ANXA1 as ANXA1 does not form a 2D network on model membranes [23,24].

Two-step FRET experiments were performed according the following scheme: the first step – FRET between ANXA5-A488 (donor 1) & ANXA5-A546 (acceptor 1), the second step – FRET between ANXA5-A546 (acceptor 1=donor 2) & ANXA5-A647 (acceptor 2) [25]. The emitted signal of ANXA5-A488 was measured in the FL1 channel with a 525/39 BP filter, ANXA5-A546 - in FL2 with a 575/29 BP filter and ANXA5-A647 - in FL3 with a 675/45 BP filter. Different combinations of labels were tested to optimize the detector and compensation settings, as the combination (ANXA5-A488+ANXA5-A647) should not give FRET and both ANXA5-A546 and ANXA5-A647 should not be excited by the 488 nm laser. A FRET signal was measured upon excitation of ANXA5-A488 (donor 1) with the 488 nm laser and the emitted signal of ANXA5-A647 (acceptor 2) was used as readout. To prevent calcium phosphate crystal formation in the flow cell, we used TBS buffer. Data analysis was performed using CXP analysis version 2.2 software (Beckman Coulter).

Confocal FRET experiments

FRET experiments were performed with the A546/A647 and Cy3/Cy5 pairs of fluorophores on a LSM 510 Meta confocal laser-scanning microscope (CLSM) (Carl Zeiss GmbH, Jena, Germany) with a Plan-Apochromat 63X/1.4

oil immersion objective at RT (21°C). The donor A546 (or Cy3) and the acceptor A647 (or Cy5) were excited with the HeNe 543 nm and HeNe 633 nm laser line and emission was sampled with a 565-615 nm and 650-710 nm BP filters, respectively. FRET channel images were obtained by excitation of the donor with the 543 nm laser and collection of emission at 650-710 nm. Images were acquired in multi track configuration mode as 8-bit images of 512×512 pixel sizes. Four time-scan averages were used to increase the signal to noise ratio. Each channel had the same optical slice (1 µm thick). During all acquisition sessions, the same microscope settings were used. The images were analyzed using ImageJ version 1.43n software (U.S. National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/) containing the "FRET and colocalization Analyzer" plug-in [26]. The "FRET index" images were generated using the equation of Youvan D.C. et al. (1997) and the mean bleed through coefficients. After thresholding (i.e. pixel intensities below a threshold value equal to 35), mean FRET efficiency values of the whole surface delimited by the cell contour were determined. The distribution of FRET pixels intensities on the cell membrane was presented as a surface plot.

Production of ANXA5-A647 labeled beads

MagnaBind Amine Derivatized Beads (Pierce) were labeled with ANXA5-A647 in the presence of the BS3 cross-linker solution following the manufacturer's instructions. In short, beads were washed three times with PBS (Braun) using a magnetic separation unit. To 5 µl washed beads, 1 ml ANXA5-A647 (50 µg mL-1 in PBS) and 1 M cross-linker suspension (BS3) were added. After incubation for 30 minutes, the remaining free amine groups were blocked with 50 µl of 1 M Tris.

Effects of unlabeled ANXA5 on the binding and on membrane-bound lattices of labeled ANXA5

To study an effect of unlabeled ANXA5 on the binding of labeled ANXA5 to ionomycin-activated platelets, unlabeled ANXA5 (20-100 nM) and 20 nM ANXA5-A546 were added simultaneously to activated cells (0.5-1.0 × 10^6 mL^{-1}) prior to the addition of calcium (2.5 mM). In binding experiments, ANXA5-A546 was excited by the 532 nm laser. To determine an effect of unlabeled ANXA5 on the ANXA5 lattice formation, activated platelets (0.5-1.0 × 10^6 mL^{-1}) were preincubated with labeled ANXA5 (ANXA5-A546+ANXA5-A647) and 2.5 mM CaCl2. Subsequently, platelets were checked for FRET prior to addition of unlabeled ANXA5 (20-100 nM). In FRET experiments, the emitted signal of the acceptor (ANXA5-A647) is shown upon excitation of the donor (ANXA5-A546) with the 532 nm laser.

Data analysis

Data are shown as mean±SD. The differences between two groups were tested by unpaired t-test (GraphPad Prism version 5.03 software). Two-sided p-values <0.05 were considered statistically significant.

RESULTS

FIX+FVIII complex formation on PS-exposing cells determined by FRET measurements

Upon binding to PS-exposing cell surfaces, coagulation factors FIX and FVIII interact with each other forming thereby a tenase complex [27,28]. We visualized FIX-FVIII interactions on the surface of ionomycin-activated COS1 cells using a flow cytometric (FCM) FRET approach. Activated COS1 cells were incubated with both Cy3-labeled FIX (donor) (**Figure 1A1**) and Cy5-labeled FVIII (acceptor) (Figure 1A2) in the presence of 2.5 mM calcium. Excitation of FIX-Cy3 by the 532 nm laser showed the emitted signal of FVIII-Cy5 (Figure 1A3) and decreased mean fluorescence intensity (MFI) of the donor (**Figure 1A4**). That indicates FRET from FIX-Cy3 to FVIII-Cy5 (fluorescence FRET signal 49.1±10.6%). The average FRET efficiency values corrected for the labeling ratios of donor and acceptor were equal to 0.25±0.07.

The existence of FIX-FVIII interactions was also confirmed by confocal FRET measurements (**Figure 1B** and **Figure S1**). Representative images illustrate the binding and co-localization of labeled coagulation factors on the surface of ionomycin-activated COS1 cells (**Figure 1B**). After correction for spectral bleed through, mean FRET values of FIX-FVIII interactions were equal to 45.4±4.8. Control experiments performed in the presence of the Ca^{2+}-chelating agent EDTA (10 mM) showed abolished binding of FIX-Cy3 and thus FRET (**Figure 1B**). The results of FRET experiments demonstrate that coagulation factors FIX and FVIII form complexes on the surface of ionomycin-activated COS1 cells.

ANXA5 but not ANXA1 efficiently inhibits FIX-FVIII interactions

To obtain more insight into the mechanism of ANXA5 anticoagulant properties, we tested an effect of unlabeled ANXA5 pre-bound on the surface of ionomycin-activated COS1 cells on FIX-FVIII interactions as well as on FIX, FVIII binding individually (**Figure 2**). ANXA5 effects were compared with those of ANXA1, which is also known to shield anionic phospholipids. FCM FRET experiments demonstrated that FIX-FVIII interactions were already inhibited by pre-bound ANXA5 at low concentrations (5 nM) (fluorescence FRET signal 4.1±1.1% versus 49.1±10.6% in the absence of ANXA5; $p<0.01$). At a saturating concentration (20 nM), ANXA5 completely blocked the interaction between FIX and FVIII (Figure 2A and Figure S2). By contrast, pre-assembled ANXA1 at 5 nM and 20 nM partly affected FIX-FVIII interactions, reducing FRET signal from 49.1±10.6% to 23.0±1.0% and 20.7±7.7%, respectively. FIX-FVIII interactions were strongly inhibited at high ANXA1 concentrations (100 nM) (**Figure 2A** and **Figure S2**).

Pre-bound ANXA5 shielding PS reduced the binding of FIX and FVIII to ionomycin-activated COS1 cells in a concentration-dependent manner (Figure 2B and Figure S2). In comparison with its effect on FIX-FVIII interactions, higher ANXA5 concentrations were required to inhibit the binding of FIX and FVIII to the cell surface. At saturating ANXA5 concentrations (20 nM), the

binding of coagulation factors was still partly reduced. Pre-bound ANXA1 (5-100 nM), even at the highest dose tested, had little inhibitory effect on FIX and FVIII binding (data not shown).

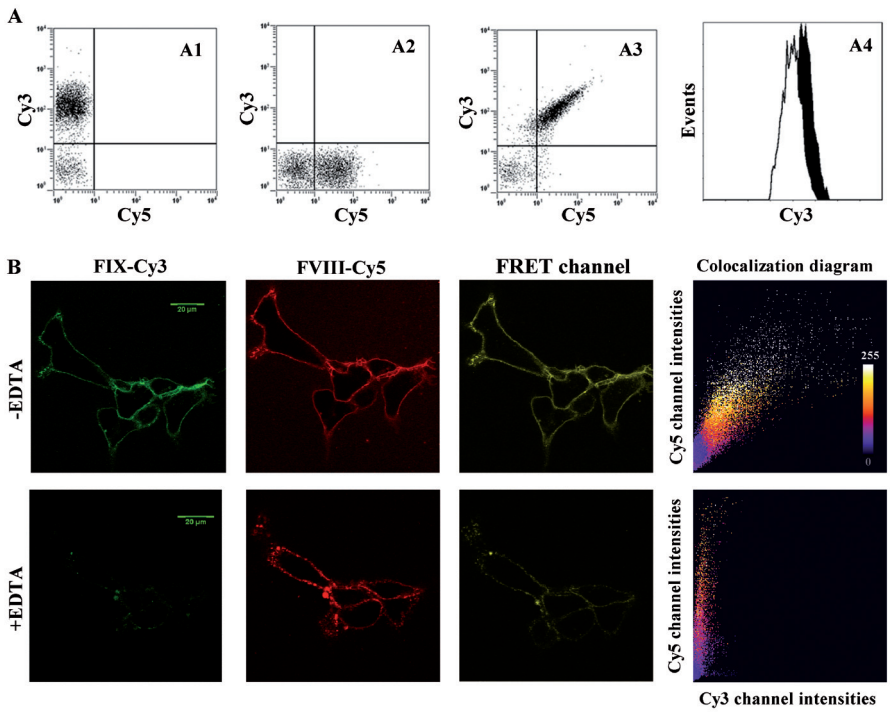

Figure 1. FIX-FVIII interactions on the surface of ionomycin-activated COS1 cells. A, For FCM measurements, activated cells were incubated with FIX-Cy3 (220 nM) and/or FVIII-Cy5 (39 nM) in the presence of 2.5 mM calcium for 15 min. Dot plots show FIX (A1) and FVIII (A2) binding to activated cells upon excitation with the 532 nm and 633 nm laser, respectively. Tenase complex formation is illustrated by energy transfer from the excited FIX-Cy3 to FVIII-Cy5 upon excitation with the 532 nm laser (A3) and decreased MFI of the donor (A4; white histogram). The black histogram represents the MFI of the FIX-Cy3 alone in the donor channel. B, Confocal images are shown of FIX-Cy3 (150 nM; green) and FVIII-Cy5 (19.5 nM; red) fluorescence intensities with the donor channel and acceptor channel setting, respectively as indicated in Materials and Methods. The uncorrected FRET signal (yellow) illustrates the emitted signal of FVIII-Cy5 upon excitation with the laser of the donor. The "Colocalization diagram" indicates co-localization of coagulation factors on the membrane of COS1 cells and the mean of FRET indices. Control experiments were performed in the presence of the Ca^{2+}-chelating agent EDTA (bottom panel). Scale bar 20 µm.

Taken together, ANXA5 but not ANXA1 efficiently inhibits FIX-FVIII interactions suggesting that ANXA5 organization on cells exposing PS is responsible for ANXA5 anticoagulant properties. Additionally, pre-bound ANXA5 interferes in the tenase complex formation not only by competing with coagulation factors for PS-binding sites but also likely by reducing the lateral mobility of membrane-bound FIX/FVIII.

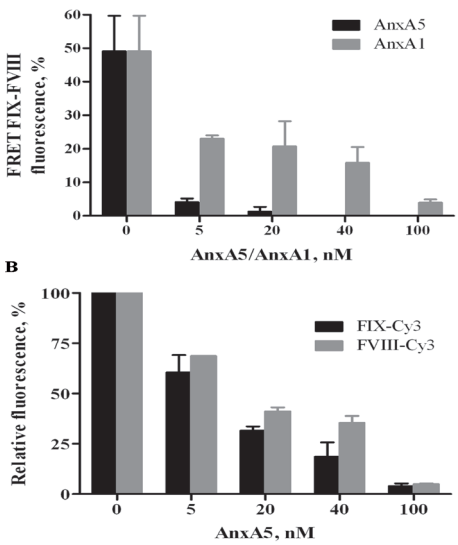

Figure 2. ANXA5 membrane-bound lattices inhibit the tenase complex formation on the surface of ionomycin-activated COS1 cells. A, Effects of pre-bound ANXA5 (0-100 nM) and ANXA1 (0-100 nM) on FIX (220 nM)-FVIII (39 nM) interactions. The emitted signal of FVIII-Cy5 (acceptor) is shown upon excitation of the donor (FIX-Cy3) with the 532 nm laser. B, A concentration-dependent reduction of FIX-Cy3 (220 nM) and FVIII-Cy3 (39 nM) binding by pre-bound unlabeled ANXA5 (0-100 nM). The graph depicts the relative fluorescence (%) as compared with the MFI of membrane-bound FIX/FVIII in the absence of ANXA5. Data are mean±SD (n=3).

ANXA5 assembles into 2D lattices on artificial membranes

Based on the suggestion that the formation of ANXA5 lattices on cell membranes could be relevant in ANXA5-based anticoagulation, we aimed to explore ANXA5 organization on PS-exposing cell surfaces. For that, we used a FRET experimental approach as labeled ANXA5 molecules are located in close proximity (less than 10 nm) on the cell membrane i.e. at a distance of one ANXA5 molecule (an average diameter 5 nm) [6] and thus could directly interact with each other [29].

ANXA5 clustering was first studied on the surface of phospholipid bilayers consisting of 20/20/60 mol% PS/PE/PC as a simplified model of the cell membrane. ANXA5 was labeled with A488, A546, A647 and purified to get the labeled proteins with 1:1 stoichiometry (**Figure S3**). Binding experiments performed by FCM showed similar Ca^{2+}-dependent binding characteristics of labeled ANXA5 moieties (0-30 nM) (**Figure S4**). FCM FRET experiments were carried out with ANXA5-A546 and ANXA5-A647 as donor and acceptor, respectively. Addition of 2.5 mM calcium to a mixture of 5 µM multilamellar lipid vesicles (MLVs), 8 nM ANXA5-A546, 12 nM ANXA5-A647 and excitation of ANXA5-A546 by the 532 nm laser resulted in a strong fluorescent signal of ANXA5-A647 due to FRET (Figure S4). The average FRET efficiency was 0.62±0.05.

Next, we investigated whether ANXA5 organization on membrane surfaces was based on multiple ANXA5 interactions. To determine these multiple interactions, we developed a two-step FRET assay (two FRET transmissions) with ANXA5-A488 (donor 1), ANXA5-A546 (acceptor 1=donor 2) and ANXA5-A647 (acceptor 2). The two-step reaction was performed with the combination of 2 nM ANXA5-A488, 6 nM ANXA5-A546 and 4 nM ANXA5-A647 in the presence of 2.5 mM Ca^{2+} (20/20/60 mol% PS/PE/PC surface) (**Figure S4**). The existence of two energy transmissions after excitation at 488 nm (from ANXA5-A488 to ANXA5-A546 and from ANXA5-A546 to ANXA5-A647) indicates that ANXA5 organization on the surface of artificial membranes is based on multiple ANXA5 interactions.

We further tested whether ANXA5 protein-protein interactions grow in a three-dimensional direction independent of anionic phospholipids. For that, ANXA5-A647 covalently coupled to amine pro-active beads was incubated with ANXA5-A546 in the presence of calcium (**Figure S4**). No FRET signal was observed, illustrating that ANXA5 does not form three-dimensional complexes independent of negatively charged phospholipids. Similar results were also obtained in the absence of calcium. Our results indicate that ANXA5 assembles into 2D tightly-packed lattices on the surface of artificial membranes.

Studying ANXA5 lattices on PS-exposing cell membranes

The FRET assay was also applied to study ANXA5 interactions on physiological cell membranes. We performed confocaol FRET measurements to provide more information on ANXA5 clustering on the plasma membrane within a single cell (**Figure 3A-B**). FRET values corrected for spectral bleed through are represented as a "FRET index" image (in a pseudocolor scale) (Figure 3B), which displays intensities of acceptor emission due to FRET in each pixel [26]. The mean FRET efficiency values of ANXA5 interactions were equal to 60.6±6.4. However, the distribution of FRET pixel intensities was quite heterogeneous, ranging from 35 (threshold values; i.e. no or very low FRET) to 210-220 (i.e. high FRET) as shown on the surface plot for a representative image (**Figure 3C**). In contrast to ANXA5, FRET efficiency values of ANXA1 interactions were considerably low (35.3±2.1, $p<0.001$), ranging from the threshold values to 90 (**Figure 3D** and **Figure S5**). ANXA5 and ANXA1 bind to PS-binding sites on the cell surface as their binding was abolished in the presence of the Ca2+-chelating agent EDTA (10 mM) (**Figure 3A-B** and **Figure S5**).

ANXA5 interactions were also examined on the surface of ionomycin-activated platelets as platelets play a dominant role in coagulation. Ionomycin treatment induced PS exposure in nearly all cells [30] and resulted in binding of labeled ANXA5 to platelets. **Figure 4B** shows the fluorescent signal of platelets labeled with ANXA5-A546 (left dot plot) and ANXA5-A647 (middle dot plot) upon excitation with the 532 nm and 633 nm laser, respectively and a strong FRET signal generated on activated platelets by excitation with the 532 nm laser (right dot plot). The average FRET efficiency was 0.59±0.05.

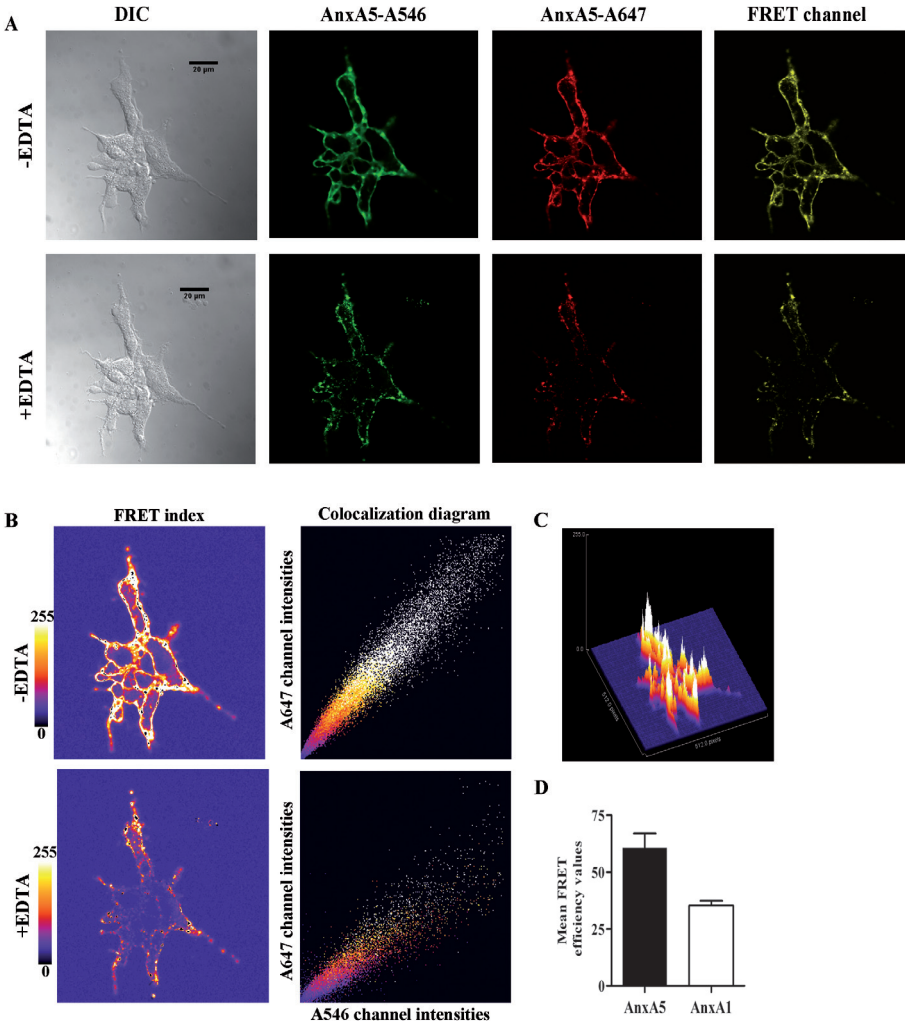

Figure 3. ANXA5 self-association on the membrane surface of ionomycin-activated COS1 cells determined by confocal FRET measurements. A, Images are shown of ANXA5-A546 (10 nM; green) and ANXA5-A647 (10 nM; red) fluorescence intensities with the donor channel and acceptor channel setting, respectively. The uncorrected FRET signal (yellow) illustrates the emitted signal of ANXA5-A647 upon excitation with the 543 nm laser. DIC indicates differential interference contrast. B, The raw FRET signal was corrected for spectral bleed through ("FRET index" image). The "Colocalization diagram" indicates co-localization of labeled ANXA5 on the membrane of COS1 cells and the mean of FRET indices. Control experiments were performed in the presence of 10 mM EDTA (A and B, lower panels). C, Surface plot illustrates the distribution of FRET pixel intensities on the cell membrane. D, Mean FRET efficiency values (error bars, SD of 43-47 cells) of ANXA5 and ANXA1 interactions ($p<0.001$). Scale bar 20 µm.

The specificity of ANXA5 binding to platelets was tested by addition of excess unlabeled ANXA5. A five-fold increase of unlabeled ANXA5 (up to 100 nM) interfered in the binding of labeled ANXA5 molecules (Figure S6). On the other hand, addition of unlabeled ANXA5 in equimolar amounts (i.e. 20 nM) to the FRET positive platelets (i.e. membrane-bound ANXA5 lattices) reduced the FRET signal but did not affect the binding of labeled ANXA5 moieties. Even increasing the concentration of unlabeled ANXA5 up to 100 nM did not affect binding of labeled ANXA5 to activated platelets (Figure S6) supporting thereby the formation of organized structures on a membrane surface. We also studied ANXA5 interactions on the surface of monocytic THP-1 cells with an apoptotic and necrotic phenotype. To control viability, THP-1 cells were labeled with 7-AAD. A high FRET signal was generated on the surface of apoptotic cells (7-AAD negative) and necrotic cells (7-AAD positive) with an average FRET efficiency of 0.48±0.04 and 0.69±0.05, respectively (**Figure 4C**). Untreated cells (7-AAD negative) gave no FRET signal. Control FRET experiments were performed with labeled ANXA1 as ANXA1 binds to PS without forming a 2D network [23,24]. After incubation of ionomycin-activated platelets with ANXA1-A546 and ANXA1-A647 and calcium (2.5 mM), a FRET signal was not observed (**Figure 5**), even after increasing the concentration of ANXA1 up to 100 nM. Summarizing, FCM and confocal FRET measurements revealed oligomerization of labeled ANXA5 on the membrane of ionomycin-activated platelets and COS1 cells as well as on apoptotic and necrotic THP-1 cells. ANXA1 membrane-bound clusters appeared to be unstructured to allow high FRET signals.

DISCUSSION

The purpose of this study was to get more insight into the mechanism of ANXA5 anticoagulant properties, especially with respect to its organization on PS-exposing cell surfaces. We aimed to prove that ANXA5 lattices inhibit the assembly formation of coagulation factors not only by competing with coagulation factors for PS-binding sites but also by reducing their lateral mobility. To test this hypothesis, we used a FRET experimental approach visualizing interactions between FIX and FVIII of the intrinsic pathway of blood coagulation as well as ANXA5 interactions on PS-exposing cell surfaces. We here provided evidence that besides the shielding of PS, ANXA5 anticoagulant activity is also based on its lattice formation on PS-exposing cells.

ANXA5 is known to interfere in blood coagulation through displacement of coagulation factors from procoagulant surfaces and thus inhibiting of their complex formation. Hence, ANXA5 is involved in this process indirectly as ANXA5 does not interact with coagulations factors [6,31]. We attempted to understand a role of ANXA5 lattices in coagulation reactions examining the influence of ANXA5 lattices on the interactions between FIX and FVIII. These membrane-bound factors interact via the A3 subunit of FVIII and the FIX light chain [27,28] forming thereby a tenase complex. Our FRET data showed that FIX-FVIII interactions on the surface of ionomycin-activated cells

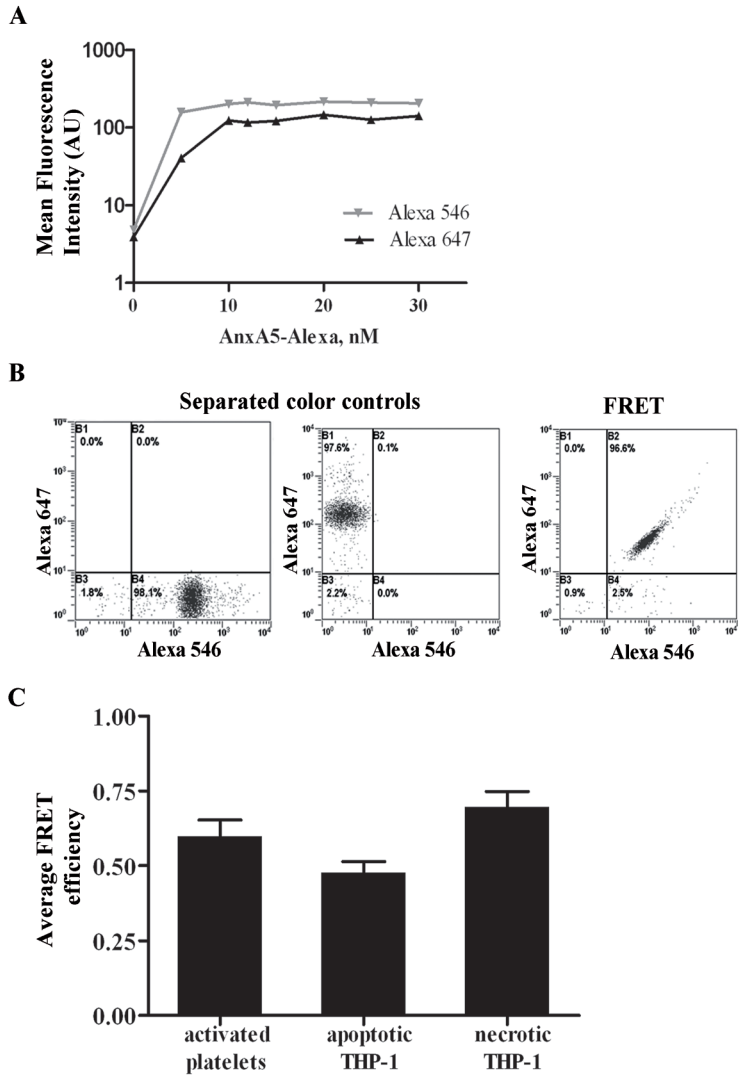

Figure 4. ANXA5 interactions on PS-exposing cell surfaces determined by FCM FRET. A, Binding of labeled ANXA5 (0-30 nM) to ionomycin-activated platelets. The results were plotted on a log10-scale y axis. AU means arbitrary units. B, Binding of ANXA5-A546 (8 nM, left dot plot) and ANXA5-A647 (12 nM, middle dot plot) to activated platelets upon excitation with the 532 nm and 633 nm laser, respectively. FRET signal (20 nM ANXA5) is shown by the emitted signal of the acceptor upon excitation of the donor with the 532 nm laser (right dot plot). Experiments (A and B) were carried out with 1.0-2.0 × 10^5 platelets in the presence of 2.5 mM Ca^{2+}. C, Average FRET efficiencies of ANXA5 interactions on ionomycin-activated platelets, apoptotic and necrotic THP-1 cells (mean ± SD). Data are from at least three independent experiments.

were affected in a different manner by membrane-bound arrays of ANXA5 and ANXA1. An inhibitory effect of pre-bound ANXA5 was already shown at low concentrations (5 nM). However, the binding of FIX and FVIII was still observable under these conditions. Pre-assembled ANXA1, by contrast, interfered in the tenase complex formation only at high concentrations. It is important to note that ANXA5 binding to phospholipid vesicles results in a rigidification of the membrane even at the mitochondrial level [32,33]. The ternary complexes ANXA5-Ca^{2+}-phospholipid are known to cause strong immobilization of the phospholipid headgroups creating regions of diminished fluidity and hence, restricting the lateral diffusion of membrane

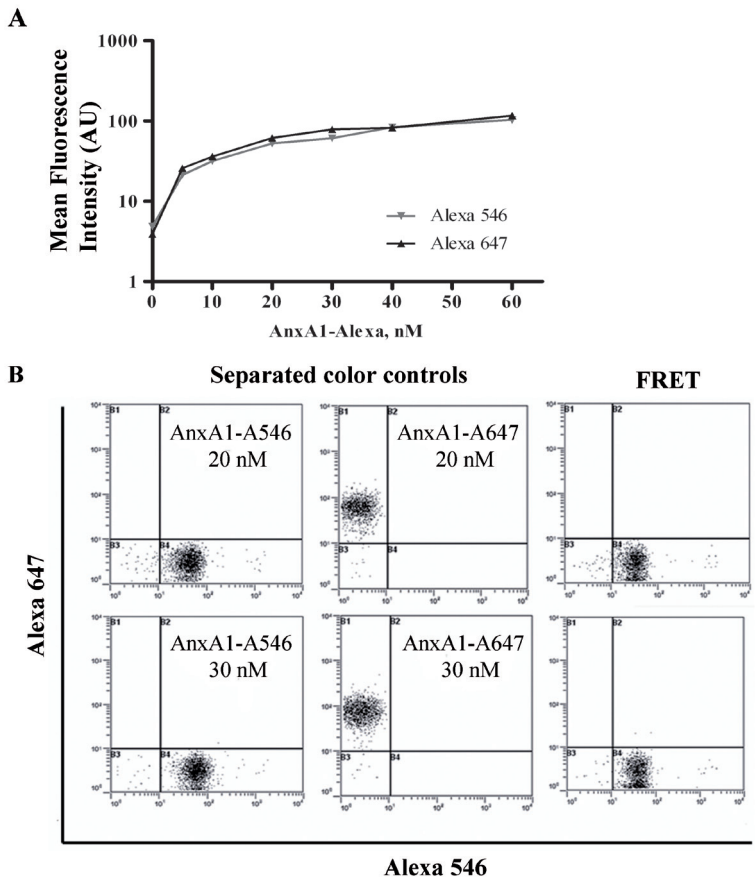

Figure 5. ANXA1 interactions on the surface of ionomycin-activated platelets determined by FCM FRET. A, Binding of labeled ANXA1 (0-60 nM) to activated platelets in the presence of 2.5 mM Ca^{2+}. The results were plotted on a log10-scale y axis. AU means arbitrary units. B, FRET was generated after addition of 2.5 mM Ca^{2+} to a mixture of activated platelets and labeled ANXA1 ((ANXA1-A546 20 nM+ANXA1-A647 20 nM) – upper panel; (ANXA1 A546 30 nM+ANXA1-A647 30 nM) – lower panel). No FRET signal was observed after excitation of the donor with the 532 nm laser (right dot plots). Experiments were carried out with 1.0-2.0 × 10^5 cells obtained from different donors (n=3).

proteins [34-36]. It has also been suggested that the rigidification of cellular membranes could be a physiological function of trimer-forming annexins (i.e. ANXA5, ANXA4 and B12) [35]. In our case, ANXA5 lattices, even at low concentrations, inhibited the interactions between FIX and FVIII likely by restricting the lateral mobility of PS-bound coagulation factors, but they did not completely block FIX/FVIII binding. ANXA1, which binds to PS without forming a 2D network, does not interact extensively with the lipid bilayer and does not immobilize the phospholipid headgroups [35]. As a result, in the presence of ANXA1 arrays, PS-bound FIX and FVIII were less restricted to interact with each other to form the tenase complex. Our data fit with the ideas of *Andree and colleagues*, who suggested that the ANXA5 anticoagulant effect could be explained by the reduced lateral transport of prothrombin and FXa in rigid sheets of ANXA5 but not only by competing with coagulation factors for PS-binding sites. Incomplete displacement of membrane-bound coagulation factors by ANXA5 as shown by *Andree et al*. [6] and in this study may be explained by the fact that ANXA5 forms open lattices. Atomic force microscopy experiments have revealed that protein-free spaces within ANXA5 lattices are large enough (~9 nм in diameter) to allow the binding of other proteins (e.g., ANXA1, ANXA2) [37,38]. In pathological situations such as in antiphospholipid syndrome, antiphospholipid antibody-mediated disruption of the ANXA5 anticoagulant shield may increase the mobility of coagulation factors and enhance their interactions ultimately leading to thrombin formation and an increased risk to develop thrombosis. Studying of ANXA5 lattice formation could be therefore of clinical importance contributing to understanding of prothrombotic properties of antiphospholipid antibodies.

To characterize ANXA5 organization on cell surfaces, we employed a FRET experimental approach, a well-known technique to study interactions of proteins within a distance of 2-10 nm. Ungethum and colleagues recently showed that ANXA5 trimerizes and assembles into 2D ordered lattices on the membrane of apoptotic Jurkat cells using a FCM FRET protocol and transmission electron microscopy analysis [16]. In our FCM FRET experiments, we observed oligomerization of labeled ANXA5 on model membranes and PS-exposing cells such as ionomycin-activated platelets and apoptotic THP-1 cells. As shown by two-step FRET measurements, the ANXA5 organization was based on multiple protein-protein interactions reflecting the tight packing of molecules. In fact, these experiments did not prove ANXA5 trimerization as the detection of FRET between three molecules (e.g., between A and B, and between B and C) does not necessarily mean the presence of a trimer [19]. However, the formation of ANXA5 organized structures was strongly supported by experiments in which addition of unlabeled ANXA5 to pre-bound labeled ANXA5 on the surface of ionomycin-activated platelets resulted in an abolished FRET signal (i.e. ANXA5-ANXA5 interactions) but not the binding of labeled ANXA5 molecules. In the light of results reported in the literature, these findings could be explained by integration of unlabeled ANXA5 within protein-free spaces of ANXA5 arrays resulting in more ordered crystal structure [35,37-39].

Confocal FRET measurements showing oligomerization of labeled ANXA5 on the cell membrane of ionomycin-activated COS1 cells further confirmed the FCM data. We additionally demonstrated that the distribution of FRET pixel intensities on the cell surface was quite heterogeneous, ranging from the threshold values (i.e. no or very low FRET) to high FRET efficiency values. It is interesting to note that "FRET index image" displays intensities of acceptor emission due to FRET in each pixel [26]. We suggest that each pixel in our setting is around 0,08 μm-square and thus represents approximately 3000 molecules ANXA5, based on the mean area per ANXA5 molecule (25.5 nm^2) [40]. Hence, clustering of ANXA5 molecules at specific domains of the plasma membrane with different PS content [41] could be an explanation for higher FRET efficiency values. Calcium-induced PS clustering [24,42] and aggregation of acidic phospholipids on apoptotic cell membranes [43,44] can indeed increase the local density of fluorescent ANXA5, subsequently leading to FRET. This phenomenon was an argument to doubt ANXA5 trimerization on a cellular membrane [45]. Ungethum et al., however, succeeded to prove that ANXA5 variant 2D1-6 lacking the ability to form trimers generates less FRET on apoptotic Jurkat cells compared with wild-type ANXA5 [16]. Another explanation for the FRET heterogeneity in our approach could be the fact that the apparent FRET is a mix of the FRET-yielding dimers, non-FRET dimers (donor-donor and acceptor-acceptor), non-oligomerized monomers and high-order oligomers [46,47]. Oligomers larger than dimers are known to result in higher FRET efficiency than dimers alone, as calculated for the mGATI transporter constructs [47]. The possibility of ANXA5 to self-assembly into large lattices is also supported by FRAP (Fluorescence Recovery After Photobleaching) experiments, which demonstrate very weak mobility of ANXA5 molecules upon binding to ionomycin-treated N1E-115 cells [48].

In contrast to ANXA5, ANXA1 generated no FRET upon binding to ionomycin-activated platelets as shown by FCM FRET experiments. Confocal FRET measurements, however, were sensitive enough to detect small patches of ANXA1 on the surface of ionomycin-activated COS-1 cells. ANXA1 appeared to form disordered clusters on the cell surfaces, which is in good agreement with previously reported findings [23,24,48]. Altogether, our results clearly showed differences in the organization of ANXA5 and ANXA1 on PS-exposing cells as well as the importance of ANXA5 lattices in inhibition of the tenase complex formation.

Several limitations to this study need to be acknowledged. At first, in our approach the 22 lysine residues present in ANXA5 molecules were labeled randomly, depending on their availability. Due to the random positioning of the fluorophores and consequently varied distances between the dye pairs, a proper critical distance calculation on basis of the FRET efficiency is not possible. Another limitation of the study concerns the functional experiments (i.e. FXa or thrombin formation) we should perform to strength the conclusion about ANXA5-induced inhibition of assembly of the tenase complex. We did not present these data since results of functional experiments to prove the influence of ANXA5 on the prothrombinase, tenase and tissue factor-FVIIa

complexes on both artificial membranes and cell surfaces have already been published by many researches [5-7]. Lastly, we used ionomycin-treated cells as stimulation with ionomycin induces PS exposure in nearly all cells [30]. It should be interesting to investigate the effect of other cell activators in our FRET approach.

To summarize, we conclude that ANXA5 lattice formation on cell surfaces exposing negatively charged phospholipids is also responsible for ANXA5 anticoagulant properties. This lattice does not fully block binding of coagulation factors but inhibits the tenase complex formation (i.e. FIX-FVIII interactions) by reducing the lateral mobility of coagulation factors. Our results as well as our FRET approach might be helpful in further understanding of thrombotic disorders where ANXA5 is involved.

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Conflict of interest
The authors state that they have no conflict of interest.

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SUPPLEMENT

Flow cytometric FRET measurements

An average FRET efficiency was calculated by quenching of the donor (E (*quenching*)) using the following formula [1]:

$$E\ (quenching) = 1 - \frac{mean\ (I_{don,acc})}{mean\ (I_{don})}$$

where $I_{don,acc}$ is the average fluorescence intensity of the donor and acceptor labeled sample, measured in the donor channel and I_{don} is the average fluorescence intensity of the donor only labeled sample, measured in the same channel.

The energy transfer efficiencies (E) of FIX-FVIII interactions were corrected for the labeling ratios ($L_R = L_D/L_A$) of donor (L_D) and acceptor (L_A) according the following equations [1,2]:

$$E_{norm} = \frac{A_{norm}}{1 + A_{norm}}$$

$$A_{norm} = \frac{L_R}{L_{Rnorm}} \times \frac{E}{1 - E}$$

where L_{Rnorm} = 1 and E_{norm} - the normalized energy transfer efficiencies.

Two-step FRET experiments:
The energy transfer efficiencies (E) of the first and the second step were determined by quenching of the donor 1 and donor 2, respectively. The two-step FRET relay efficiency is as follows: $E_{relay} = E_{first\ step} \times E_{second\ step}$ [3].

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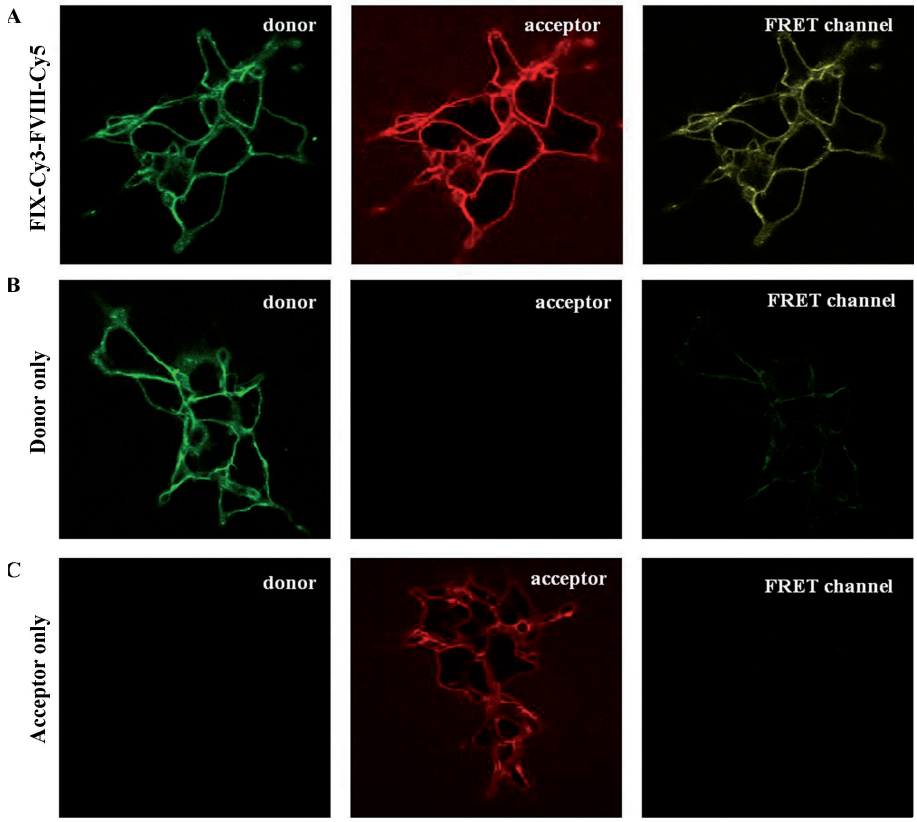


Figure S1. FIX-FVIII interactions on the surface of ionomycin-activated COS1 cells. The acceptor-sensitized emission FRET on activated COS1 cells labeled with the FRET-pair (FIX-Cy3 and FVIII-Cy5) (A), the donor-only (FIX-Cy3) (B) and the acceptor-only (FVIII-Cy5) (C) is shown. B, donor spectral bleed through is observed in the FRET channel after excitation with the donor laser (543 nm), whereas there is no crosstalk in the acceptor channel by excitation with the laser of acceptor (633 nm). C, acceptor spectral bleed through detected in the FRET channel after excitation with the donor laser is low.

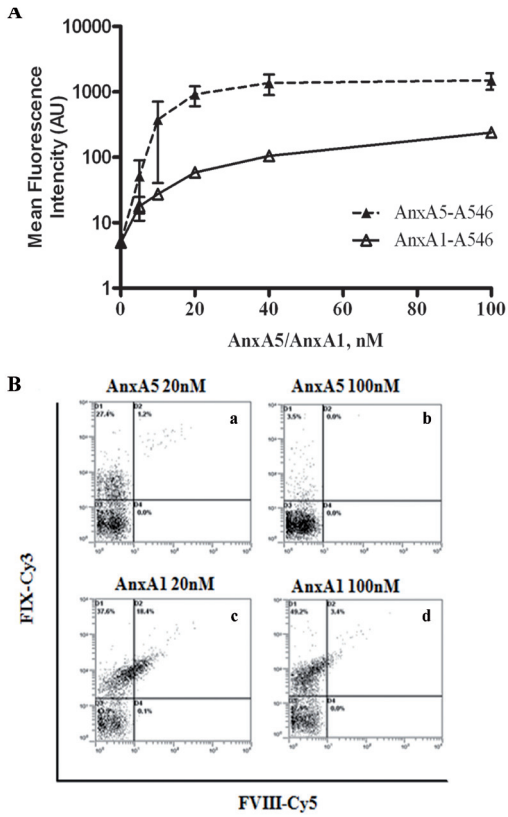


Figure S2. Membrane-bound arrays of ANXA5 and ANXA1 affect FIX-FVIII interactions on the surface of ionomycin-activated COS1 cells.
A, Binding of ANXA5 and ANXA1 to activated COS1 cells. ANXA5-A546 and ANXA1-A546 were excited by the 532 nm laser. Data are mean±SD (n=3). B, Dot plots illustrate abolished FIX-FVIII interactions by 20 nM of pre-bound unlabeled ANXA5 (a) and 100 nM of pre-bound unlabeled ANXA1 (d). ANXA5 at 100 nM completely interfered in the binding of coagulation factors (b). The emitted signal of FVIII-Cy5 (acceptor) is shown upon excitation of the donor (FIX-Cy3) with the 532 nm laser.

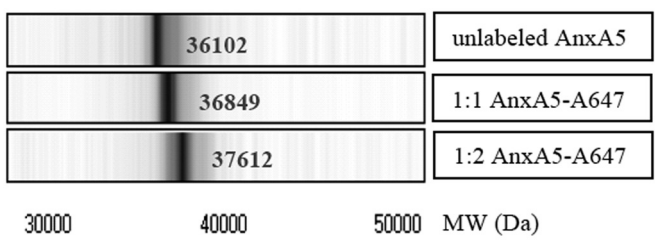


Figure S3. Stoichiometry of labeled ANXA5 after purification by FPLC. The dye to protein ratios were determined by SELDI-TOF MS and visualized by a gel view. Unlabeled ANXA5 showed a band at approximately of 36 kDa. The changes in molecular weight (MW) correspond to the number of fluorescent dye molecules covalently coupled to one ANXA5 molecule.

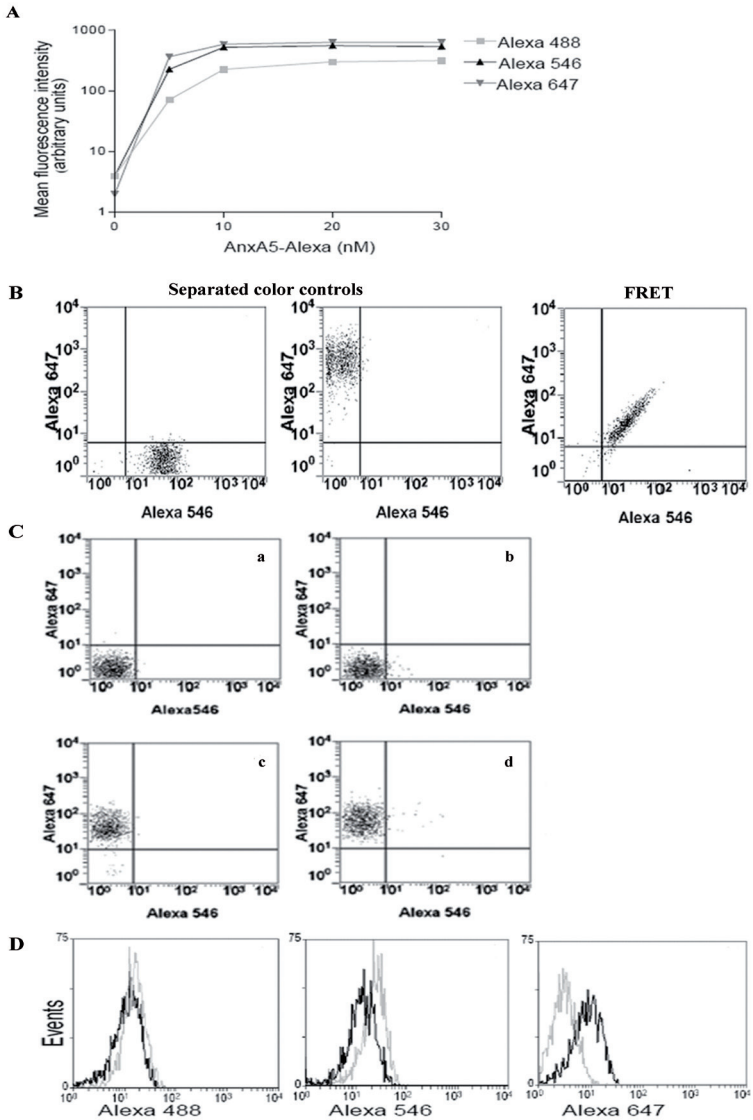


Figure S4. ANXA5 interactions on the surface of artificial membranes.
A, Binding of labeled ANXA5 (0-30 nM) to MLVs (5 μM). ANXA5-A488, ANXA5-A546 and ANXA5-A647 were excited by the 488 nm, 532 nm and 633 nm laser, respectively. The results were plotted on a log10-scale y axis. B, Binding of ANXA5-A546 (8 nM, left dot plot) and ANXA5-A647 (12 nM, middle dot plot) upon excitation with the 532 nm and 633 nm laser, respectively. FRET was generated after addition of Ca^{2+} to a mixture of 5 μM MLVs and 20 nM labeled ANXA5. FRET signal is shown by the emitted signal of the acceptor (ANXA5-A647) upon excitation of the donor (ANXA5-A546) with the 532 nm laser (right dot plot). C, ANXA5 interactions are two-dimensional but not three-dimensional. ANXA5-A647 (22 nM) coupled to amine

beads was excited by the 532 nm laser (a, b) or 532 nm and 633 nm laser together (c, d). The signal of ANXA5-A647 coated beads is shown in the Alexa 647 channel (c). Addition of ANXA5-A546 (20 nM) to ANXA5-A647 coated beads did not result in a positive signal in the Alexa 546 channel (b, d) and no FRET signal was observed (b). D, Multiple ANXA5 interactions on a phospholipid bilayer measured by two-step FRET. The black curves show the MFI of the two-step FRET (ANXA5-A488+ANXA5-A546+ANXA5-A647). The grey curves illustrate the MFI of the following steps: ANXA5-A488, (ANXA5-A488+ANXA5-A546) and (ANXA5-A546+ANXA5-A647) in the Alexa 488, 546 and 647 channel, respectively. In the two-step FRET, there is a small decrease in MFI of Alexa 488 (left plot) and Alexa 546 (middle plot) and an increase in MFI of Alexa 647 (right plot). All experiments (A-D) were performed with 5 μM MLVs in the presence of 2.5 mM Ca^{2+}.

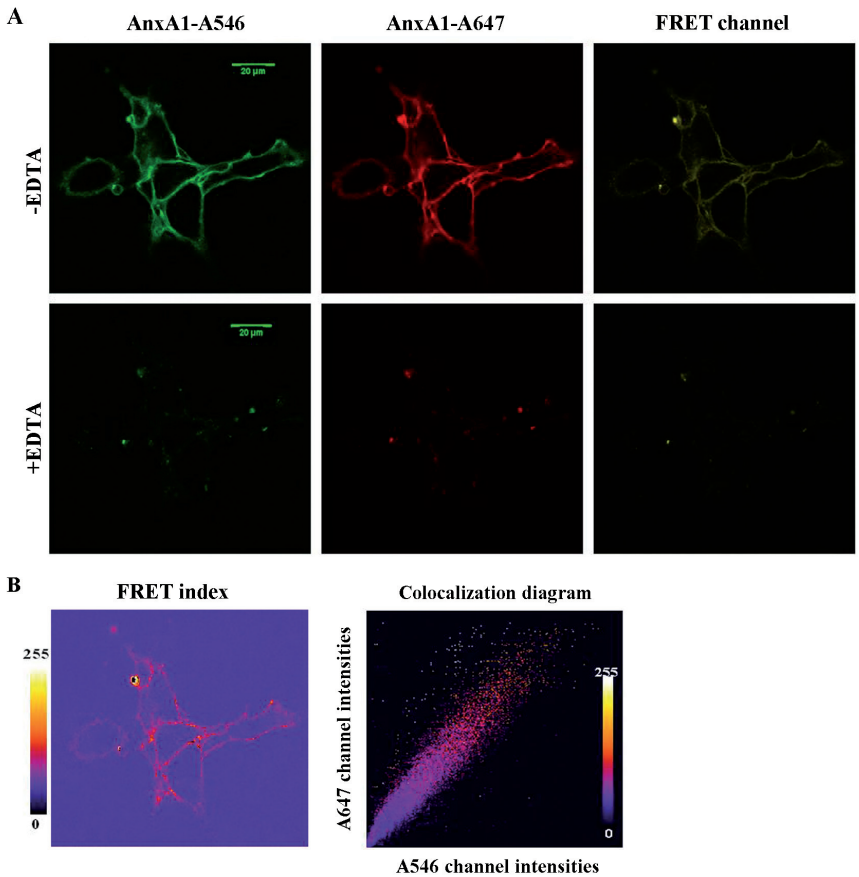


Figure S5. ANXA1 interactions on the surface of ionomycin-activated COS1 cells determined by confocal FRET measurements. A, Images are shown of ANXA1-A546 (20 nM; green) and ANXA1-A647 (20 nM; red) fluorescence intensities with the donor channel and acceptor channel setting, respectively. The uncorrected FRET signal (yellow) illustrates the emitted signal of ANXA1-A647 upon excitation with the laser of the donor (543 nm). Control experiments were performed in the presence of 10 mM EDTA (bottom panel). B, The FRET signal was corrected for spectral bleed through ("FRET index" image). The "Colocalization diagram" shows co-localization of labeled ANXA1 on the membrane of COS1 cells and the mean of FRET indices. Scale bar 20 μm.

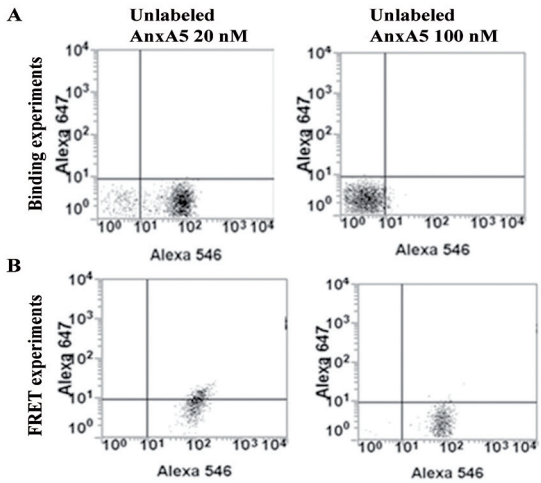
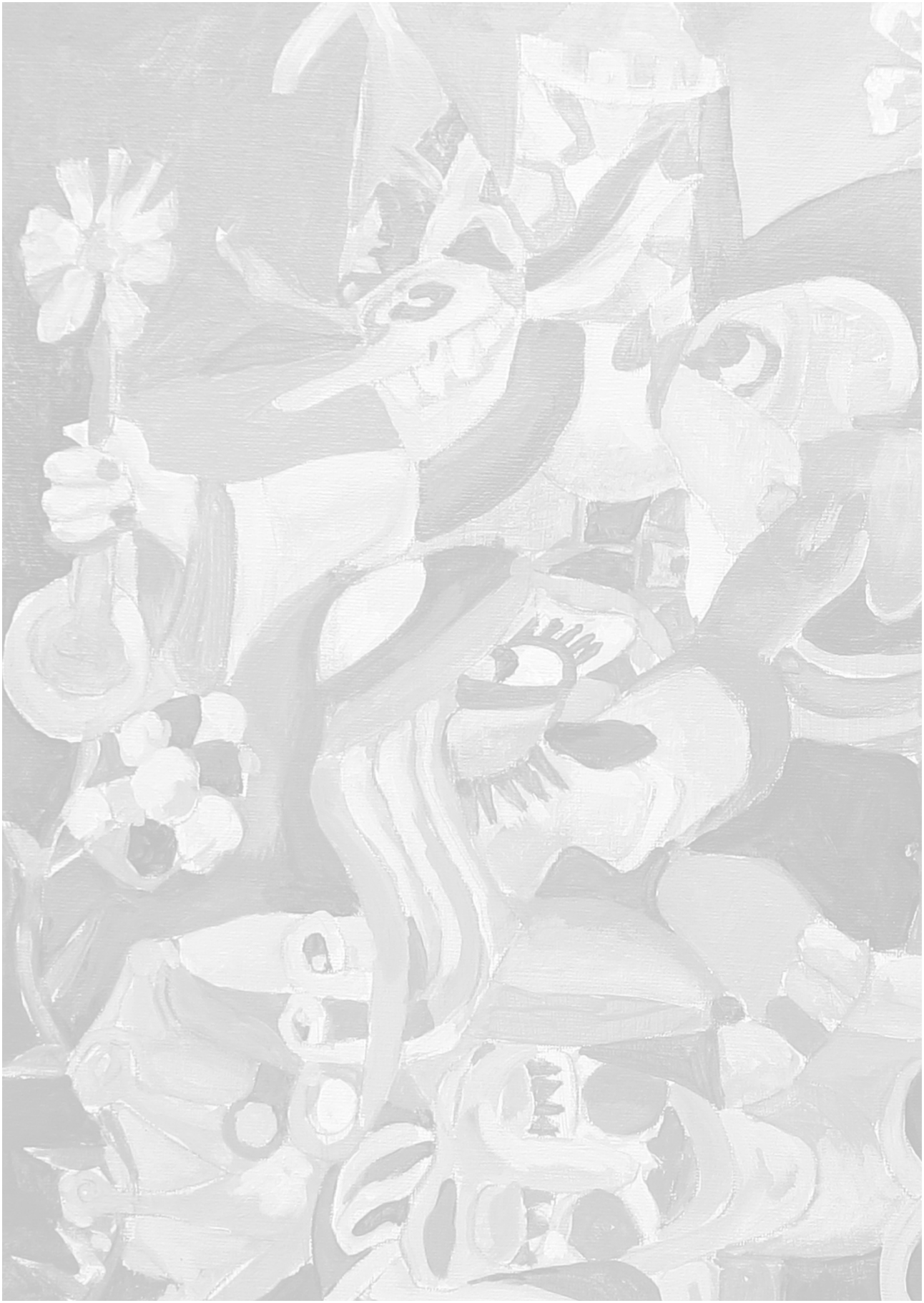


Figure S6. Effects of unlabeled ANXA5 on the binding (A) and 2D network (B) of labeled ANXA5. A, Unlabeled ANXA5 at 20 nM and 100 nM concentrations interfere in the binding of ANXA5-A546 (20 nM) to ionomycin-activated platelets. ANXA5-A546 was excited by the 532 nm laser. B, Addition of unlabeled ANXA5 (20 nM) to the membrane-bound ANXA5 lattices affected the FRET signal (left dot plot). Increasing the concentration of unlabeled ANXA5 up to 100 nM resulted in abolished FRET signal but not the binding of labeled ANXA5 (middle dot plot). The emitted signal of the acceptor (ANXA5-A647) is shown upon excitation of the donor (ANXA5-A546) with the 532 nm laser. Results are representative of at least four independent experiments with platelets obtained from different donors. Experiments were performed with 1.0-2.0 × 10^5 ionomycin-activated platelets and 2.5 mM Ca^{2+}.



Chapter 3

Polymorphisms in the *Annexin A5* gene influence circulating Annexin A5 levels in healthy controls

Larissa Hiddink, Marieke C.H. de Visser and Waander L. van Heerde

Thrombosis Research 2012; 129:815-817

Annexin A5 (ANXA5), a Ca^{2+}- and phospholipid-binding protein of the annexin family, has strong anticoagulant and antithrombotic properties as well as antiinflammatory features, both in vitro and in vivo [1-5]. Its exact physiological function, however, has not been fully understood. Circulating ANXA5 levels are low in healthy individuals (0 to 5 ng/mL) [6]. Elevated plasma ANXA5 levels reported in patients with acute myocardial infarction, severe trauma [7,8] and sickle cell disease [9] are shown to reflect the severity of cell damage. Reduced plasma ANXA5 levels are associated with the presence and extent of atherosclerotic plaques [10] as well as recurrent pregnancy loss [11].

The contribution of genetic variations to the variability of plasma ANXA5 levels is poorly understood. *Gonzalez-Conejero and colleagues* reported that the single nucleotide polymorphism (SNP) rs11575945, which is located one nucleotide upstream of the ATG initiation codon in exon 2 of the *ANXA5* gene, protects against both a first myocardial infarction and a new thrombotic event during 36 months follow-up [12,13]. The authors suggested a protective role of elevated plasma ANXA5 levels in the occurrence of arterial thrombosis. However, *Marilyn Kozak* questioned increased translation efficiency by the minor rs11575945T-allele, demonstrating its small contribution to initiation of translation [14,15], and v*an Heerde and colleagues* showed normal levels of circulating *ANXA5* in T-allele carriers [16]. Recently, Bogdanova et al. [17] described the M2 haplotype, a combination of minor alleles of four promoter SNPs (rs112782763, rs28717001, rs28651243, rs113588187). The M2 haplotype is related to reduction of *ANXA5* gene promoter activity in vitro [17] as well as ANXA5 mRNA levels in placenta ex vivo [18,19]. The presence of this haplotype is associated with thrombotic obstetric complications [17,20,21] and deep venous thrombosis [22].

Here we investigated whether *ANXA5* genetic variations influence plasma levels of ANXA5. To take into account all genetic information, a 496-bp promoter region and exon 2 of the *ANXA5* gene were sequenced in healthy individuals. Subsequently, haplotypes were inferred, and the association of common haplotypes with circulating ANXA5 levels was investigated.

The study population included 137 healthy individuals: 58 blood donors attending the Sanquin Blood bank, region South East, Nijmegen and 79 individuals recruited by Radboudumc. In accordance with the Declaration of Helsinki, written informed consent was obtained from each participant. Plasma samples were obtained by centrifugation of 3.2% citrated blood at 4200g for 10 minutes. Circulating ANXA5 levels were determined by a Zymutest ANXA5 ELISA (Hyphen Biomed) following manufacturer's instructions. Genomic DNA was isolated from peripheral leukocytes. A 496-bp fragment of the *ANXA5* promoter (261 base pairs upstream and 235 base pairs downstream of the first transcription start point) was amplified by polymerase chain reaction using two oligonucleotide primers as described [17]. Exon 2 with flanking regions (130-bp fragment) was amplified as previously described [23]. Purified amplicons were sequenced using the BigDye Terminator

Table 1. Polymorphisms and haplotypes of the *ANXA5* gene in healthy controls (n=131).

	SNP1	SNP2	SNP3	SNP4	SNP5	SNP6	SNP7	
	g.-628C>T	g.-467G>A	g.-448A>C	g.-422T>C	g.-373G>A	g.-302T>G	g.-1C>T	
rs number*	rs62319820	rs112782763	rs28717001	rs28651243	rs113588187	rs1050606	rs11575945	
MAF†	0.09	0.11	0.20	0.20	0.11	0.49	0.11	
Haplotype / Bogdanova's haplotype‡								**Haplotype frequency**
H1 / N	C	G	A	T	G	T	C	0.51
H2 / N	C	G	A	T	G	**G**	C	0.29
H3 / M2	C	**A**	**C**	**C**	**A**	**G**	**T**	0.11
H4 / M1	**T**	G	**C**	**C**	G	**G**	C	0.08
H5 / M1	C	G	**C**	**C**	G	T	C	0.004

Nucleotide numbering from the ATG initiation codon,
*according to http://www.ncbi.nlm.nih.gov/snp;
†MAF, minor allele frequency; minor alleles in bold and underlined.
‡Haplotypes (SNPs 2-5) according to Bogdanova et al. [17]: N haplotype (G-A-T-G); M2 haplotype (A-C-C-A); M1 haplotype (G-C-C-G).

v3.1 Cycle Sequencing Kit (Applied Biosystems) and analyzed on the ABI 3730 PRISM DNA Analyzer (Applied Biosystems). For statistical analyses, we used only complete data of 131 subjects. Hardy-Weinberg equilibrium for each SNP was evaluated by a chi-square test. Haploview software was used to estimate the degree of linkage disequilibrium (r^2 values) between all SNP pairs and to determine haplotypes (H) [24]. Haplotypes were assigned manually to all individuals. Skewed distributed ANXA5 levels were 10log-transformed prior to statistical analyses. Differences between the log-transformed values were tested by t-test. All values reported were reconverted to geometric means with the appropriate 95% confidence interval (CI). Two-sided P-values <0.05 were considered statistically significant. Sequence analysis of a 496-bp fragment of the *ANXA5* promoter in 67 male (mean 41.5±10.0 years, ±SD) and 64 female (mean 41.8±9.6 years, ±SD) healthy subjects confirmed the presence of four SNPs (SNP2 rs112782763, SNP3 rs28717001, SNP4 rs28651243, SNP5 rs113588187) previously reported by *Bogdanova et al.* [17]. Additionally, two other genetic variants (SNP1 rs62319820, SNP6 rs1050606) were detected. All participants were also examined for the presence of SNP7 rs11575945 in exon 2. The genotype distributions of all seven SNPs were in Hardy-Weinberg equilibrium. Haploview analysis showed a high degree of linkage disequilibrium between all variants except for SNP1 and SNP6. SNP3 and SNP4 as well as SNP2, SNP5 and SNP7 were completely linked (r^2=1). Because of the tight linkage, only four common haplotypes (frequency >1%) were present (**Table 1**).

Table 2. Association of *ANXA5* haplotypes with plasma ANXA5 levels.

Haplotype	Number (%) n=131	Geometric mean ANXA5 (95% CI),ng/mL
Haplotype 1		
H1H1	31 (23.7)	0.94 (0.82-1.07)
H1Hx	71 (54.2)	0.93 (0.84-1.02)
HxHx	29 (22.1)	0.77 (0.61-0.96)
Haplotype 2		
H2H2	10 (7.6)	0.52 (0.44-0.62)*
H2Hx	56 (42.7)	0.85 (0.76-0.94)*
HxHx	65 (49.6)	1.01 (0.91-1.13)
Haplotype 3		
H3H3	1 (0.8)	0.60
H3Hx	27 (20.6)	0.82 (0.67-1.02)
HxHx	103 (78.6)	0.92 (0.84-1.00)
Haplotype 4		
H4H4	1 (0.8)	1.56
H4Hx	21 (16.0)	1.40 (1.14-1.72)*
HxHx	109 (83.2)	0.81 (0.76-0.88)

Hx indicates all haplotypes except for the one given; CI, confidence interval.*P<0.05 as compared with non-carriers.

Plasma ANXA5 levels did not differ between male and female subjects (mean 0.88 ng/mL, 95% CI: 0.79-0.98 versus mean 0.90 ng/mL, 95% CI: 0.80-1.01). ANXA5 levels were not influenced by age (linear regression; P=0.32). Haplotypes H1 and H2, the two major haplotypes, included Bogdanova's haplotype N [17]. Interestingly, haplotype H2 was associated with decreased plasma ANXA5 levels (**Table 2**). Homozygous H2 carriers had lower ANXA5 levels (mean 0.52 ng/mL, 95% CI: 0.44-0.62) than heterozygous H2 carriers (mean 0.85 ng/mL, 95% CI: 0.76-0.94) and non-H2 subjects (mean 1.01 ng/mL, 95% CI: 0.91-1.13). Since H1 and H2 were only discriminated by SNP6, it is suggestive that the G-allele of this SNP is responsible for the decreased ANXA5 levels. Indeed, homozygous GG carriers had slightly lower ANXA5 levels (mean 0.76 ng/mL, 95% CI: 0.60-0.96) than TG heterozygotes (mean 0.92 ng/mL, 95% CI: 0.84-1.02) and TT carriers (mean 0.94 ng/mL, 95% CI: 0.82-1.07) (P>0.05) (Table S1). However, the rs1050606G-allele was also present in haplotypes H3 and H4, which were not associated with decreased ANXA5 levels. The contribution of the G allele to a reduction of plasma ANXA5 needs to be further studied since the rs1050606G-allele is recently identified as a potential novel risk factor for recurrent pregnancy loss in the Japanese population [21].

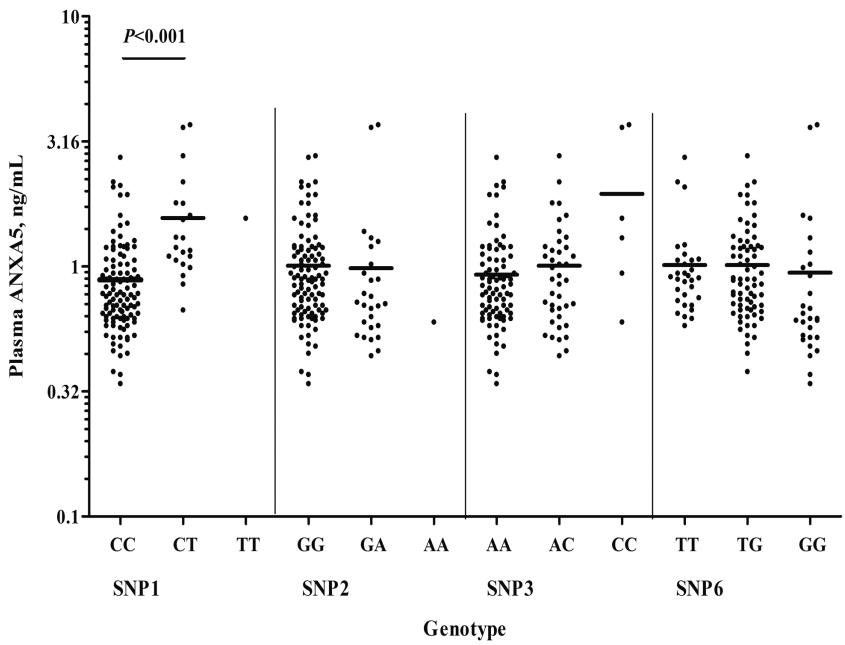


Figure 1. Association of individual *ANXA5* SNPs with plasma ANXA5 levels. Due to complete linkage between SNP2 rs112782763, SNP5 rs113588187 and SNP7 rs11575945 as well as between SNP3 rs28717001 and SNP4 rs28651243, only the results for SNP2 genotypes and SNP3 genotypes, respectively, are shown. The results were plotted on a log10-scale y axis. Horizontal lines represent means. The means of log-transformed ANXA5 levels for different groups were compared by t-test.

Another important result was the association of haplotype H4 with increased levels of ANXA5. H4 heterozygotes had significantly higher circulating ANXA5 levels than non-H4 carriers (mean 1.40 ng/mL, 95% CI: 1.14-1.72 versus mean 0.81 ng/mL, 95% CI: 0.76-0.88, respectively, P<0.001). Haplotype H4 is an extension of the M1 haplotype [17]. The possession of the minor T-allele of SNP1 rs62319820, which was unique for haplotype H4, seemed to be a major contributor to higher plasma ANXA5 levels (**Figure 1**, **Table S1**). Our results contradict findings of *Bogdanova et al.* who demonstrated reduced *ANXA5* promoter activity for the M1 haplotype [17]. The absence of the rs62319820T-allele in their luciferase reporter constructs could be one of the reasons for this contradiction.

Haplotype H3 was the third major haplotype in our population. Circulating ANXA5 levels in H3 heterozygotes (mean 0.82 ng/mL, 95% CI: 0.67-1.02) tended to be lower compared with those in non-H3 carriers (mean 0.92 ng/mL, 95% CI: 0.84-1.00) (P=0.27). In line with our previous finding [16], the minor rs11575945T-allele (SNP7) did not significantly influence ANXA5 levels (**Figure 1, Table S1**). Haplotype H3 is an extension of the M2 haplotype. Although the presence of the M2 allele of *ANXA5* was found to be associated with decreased ANXA5 mRNA levels in placenta ex vivo [18,19], no correlation was observed with protein levels in the same samples [19]. In the present study, we showed no clear evidence for decreased plasma ANXA5 levels in H3 carriers. However, since the M2 haplotype of *ANXA5* is associated with placental thrombosis [17,20,21] as well as deep venous thrombosis [22], slightly lower plasma ANXA5 levels found in H3 carriers need more attention and further investigation in larger studies.

In conclusion, we reported that *ANXA5* haplotypes may determine the variability of plasma ANXA5 levels in healthy subjects. More research is warranted to confirm our findings. Clarifying the relevance of the variability of circulating ANXA5 levels may be of clinical importance.

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Conflict of interest
The authors state that they have no conflict of interest.

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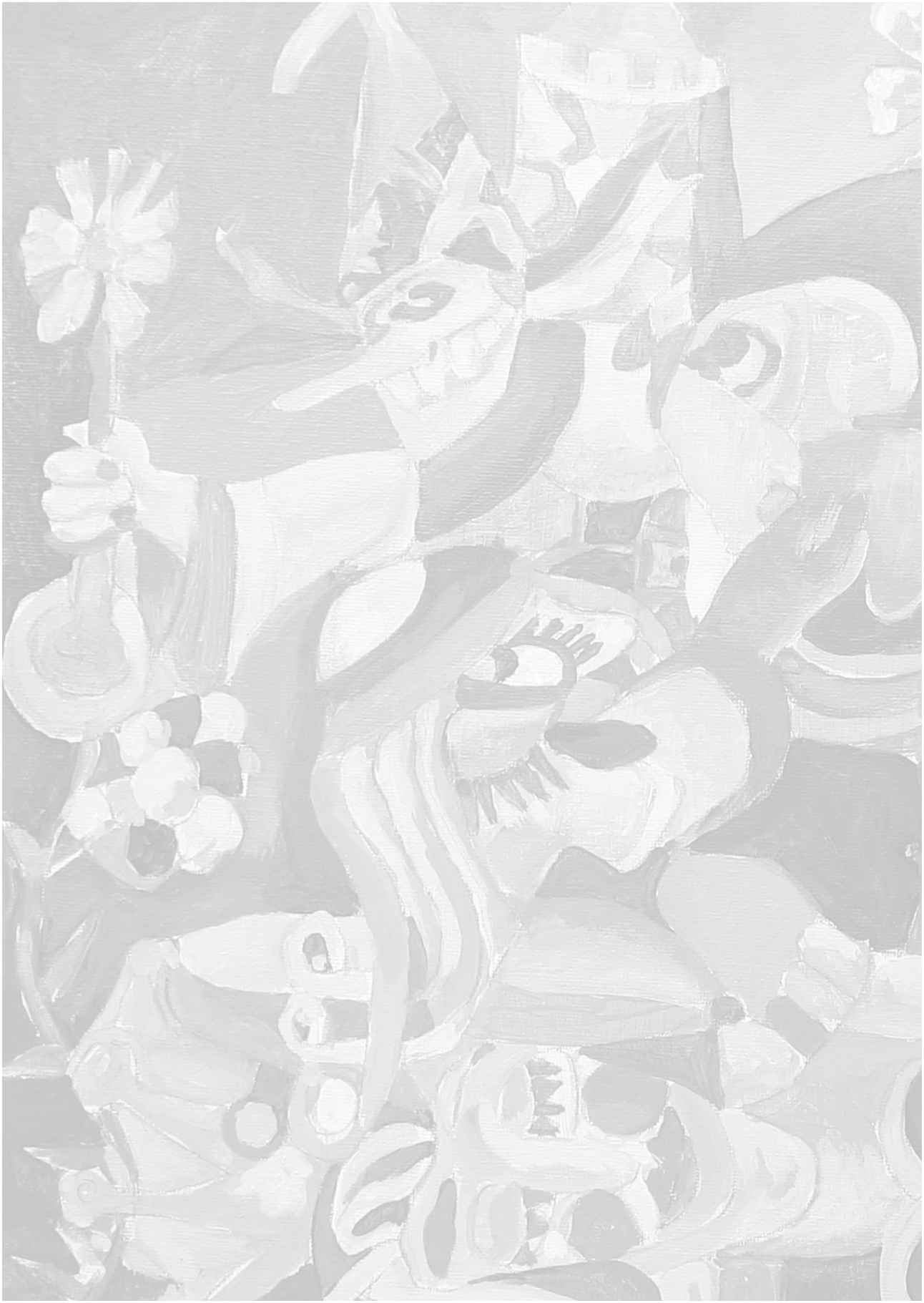
Table S1. Plasma ANXA5 levels in different genotype groups.

Polymorphism	Genotype	Number (n=131)	Geometric mean ANXA5 (95% CI), (ng/mL)
SNP1 rs62319820	CC	109	0.81 (0.76-0.88)
	CT	21	1.40 (1.14-1.72)*
	TT	1	1.56
SNP2 rs112782763	GG	103	0.92 (0.84-0.99)
	GA	27	0.82 (0.67-1.02)
	AA	1	0.6
SNP3 rs28717001	AA	84	0.85 (0.78-0.93)
	AC	41	0.91 (0.79-1.05)
	CC	6	1.57 (0.73-3.38)
SNP4 rs28651243	TT	84	0.84 (0.77-0.92)
	TC	41	0.91 (0.79-1.05)
	CC	6	1.57 (0.73-3.38)
SNP5 rs113588187	GG	103	0.92 (0.84-0.99)
	GA	27	0.82 (0.67-1.02)
	AA	1	0.6
SNP6 rs1050606	TT	31	0.94 (0.82-1.07)
	TG	72	0.92 (0.84-1.02)
	GG	28	0.76 (0.60-0.96)
SNP7 rs11575945	CC	103	0.92 (0.84-0.99)
	CT	27	0.82 (0.67-1.02)
	TT	1	0.6

CI means confidence interval.
*P<0.001 as compared with the wild-type.



Chapter 4

Annexin A5 haplotypes in familial hypercholesterolemia: lack of association with carotid intima-media thickness and cardiovascular disease risk

Larissa Hiddink, Geesje M. Dallinga-Thie, G. Kees Hovingh, Marieke C.H. de Visser, Petronella G.M. Peer, Anton F.H. Stalenhoef, Waander L. van Heerde

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ABSTRACT

Objective: Annexin A5 (ANXA5) has been suggested to possess antiatherogenic properties. We investigated whether *ANXA5* genetic variations and plasma ANXA5 levels were associated with carotid atherosclerosis and contributed to cardiovascular disease (CVD) risk in patients with familial hypercholesterolemia (FH).
Methods: We sequenced the promoter region and exon 2 of *ANXA5* in 284 FH patients from the ASAP (Atorvastatin versus Simvastatin on Atherosclerosis Progression) trial. Common haplotypes (H) were constructed based on seven single nucleotide polymorphisms (SNPs). We studied whether plasma ANXA5 levels or *ANXA5* haplotypes were associated with the extent of atherosclerosis (evaluated by carotid intima-media thickness (IMT). The association between *ANXA5* haplotypes and the risk for CVD events was investigated in 1730 FH patients from the GIRaFH (Genetic Identification of Risk factors in Familial Hypercholesterolemia) study.
Results: In ASAP, individuals carrying the *ANXA5* haplotype H2 exhibited lower plasma ANXA5 levels, whereas H4 carriers had increased levels of circulating ANXA5 compared to non-carriers. Plasma ANXA5 levels were not associated with carotid IMT. None of the four *ANXA5* haplotypes correlated with the age-related IMT progression (ASAP study) or contributed to CVD risk (GIRaFH cohort).
Conclusions: Both *ANXA5* haplotypes and plasma ANXA5 levels were not associated with carotid IMT progression or CVD risk in FH patients.

INTRODUCTION

Annexin A5 (ANXA5), a calcium-dependent phospholipid-binding protein, is highly expressed by endothelial cells [1] and present in atherosclerotic plaques, especially at sites with high prothrombotic potential [2;3]. ANXA5 is considered to have antithrombotic capacities, by virtue of its scavenging effect on anionic phospholipids and downregulation of the surface expression of tissue factor [4-7]. Since atherosclerotic vessel walls contain apoptotic and activated cells that expose negatively charged phospholipids [8;9], ANXA5 may form an antithrombotic shield on these cell surfaces, thereby restricting their tendency to induce thrombus formation. Apart from its antithrombotic property, ANXA5 has also been shown to exert potent anti-inflammatory activities, which are attributed to downregulation of interferon-gamma (IFN-γ)-mediated inflammatory cellular responses [10]. In addition, ANXA5 inhibits phospholipase A2 activity, an enzyme essential for the generation of pro-inflammatory mediators [11;12]. Concomitantly, it has been shown that ANXA5 binds to oxidized low-density lipoprotein (oxLDL) thereby preventing the ox-LDL induced procoagulant and pro-inflammatory effects [13]. In proatherogenic ApoE-/- mouse models, it has been demonstrated that ANXA5 administration reduces local inflammation and vascular remodeling as well as improves vascular function, confirming the notion that ANXA5 has antiatherogenic effects [14;15].

Additionally to the biochemical properties of ANXA5, attention is paid to a number of single nucleotide polymorphisms (SNPs) in *ANXA5* but so far, little is known about their relative contribution to cardiovascular disease (CVD) risk. The minor T-allele of rs1131239, which is located in the Kozak sequence (i.e., one nucleotide upstream of the ATG initiation codon) in exon 2 of *ANXA5*, is associated with a decreased risk of myocardial infarction under the age of 45 [16] and a lower risk of a new thrombotic event during 36 months follow-up [17]. Increased plasma ANXA5 levels in rs1131239T-allele carriers were found to be protective in the occurrence of arterial thrombosis. Subsequent studies, however, were unable to replicate these findings [18;19]. Moreover, *ANXA5* intronic SNPs rs4833229 and rs6830321 are associated with increased restenosis risk in patients undergoing percutaneous coronary intervention for atherosclerosis [15]. Recently, *Bogdanova and colleagues* described the three *ANXA5* promoter haplotypes (N, M1 and M2) and found that the *ANXA5* M2 haplotype consisting of the minor alleles of four SNPs (rs112782763, rs28717001, rs28651243, rs113588187) reduces *ANXA5* promoter activity in a luciferase reporter gene assay [20]. While the *ANXA5* M2 haplotype has been linked to thrombotic obstetric complications [20-22], its contribution to CVD risk remains to be determined. More recently, we demonstrated that plasma ANXA5 levels in healthy individuals are affected by genetic variants in *ANXA5*. Haplotype H2 was associated with significantly decreased plasma ANXA5 levels whereas haplotype H4 was associated with increased plasma ANXA5 levels [23]. Our defined haplotypes H3 and H4 are extensions of the M2 and M1 haplotypes, and haplotypes H1 and H2 include the wild-type haplotype N described by *Bogdanova et al.* The clinical relevance of plasma ANXA5 levels on

atherosclerotic CVD burden is yet unclear. Hypothetically, higher plasma ANXA5 levels are expected to have a protective role, whereas lower ANXA5 levels are likely to be associated with progression of atherosclerosis (i.e., an increased plaque formation and an increased CVD risk). In this context, it has been shown that circulating ANXA5 levels correlate inversely with the severity of angiographically determined coronary stenosis and the extent of atherosclerotic plaque formation [24].

In this study, we set out to test the hypothesis that plasma ANXA5 levels and *ANXA5* genetic variations are associated with carotid atherosclerosis and contribute to CVD risk in patients with familial hypercholesterolemia (FH), an autosomal dominant disease characterized by high plasma levels of low-density lipoprotein cholesterol (LDL-C) and an increased CVD risk.

MATERIALS AND METHODS

ASAP trial

ASAP (Atorvastatin versus Simvastatin on Atherosclerosis Progression) was a randomized, double-blind, two-center (Amsterdam and Nijmegen) study [25]. A total of 325 FH patients aged 30-70 years participated in the ASAP study. Eligibility of the patients was based on plasma LDL-C levels (>5.5 mmol/L) and the absence of significant clinical, hematological and biochemical abnormalities. Exclusion criteria were coronary heart disease within previous 3 months, hypertension, secondary hyperlipidemia, diabetes and other endocrine diseases [25]. The Ethics Committees of both trial centers approved the study. In the present study, we used only data collected after an 8-week placebo run-in period before starting any intervention with statins. Carotid IMT measurements were performed as described [25]. Briefly, ultrasound examinations were performed using a Biosound Phase-2 real time scanner (BiosoundEsaote, USA) equipped with a 10 MHz transducer. Three 10 mm segments were scanned bilaterally: the distal portion of the common carotid artery (CCA), the carotid bifurcation (BUL) and the proximal portion of the internal carotid artery (ICA). Both near and far walls were evaluated. Mean carotid IMT was calculated as averaged over anterior and posterior walls in the CCA, BUL and the posterior wall of the ICA, bilaterally (i.e. the mean from available 10 sites). Of the 325 participants, genomic DNA of 299 patients was available for sequencing in this study.

GIRaFH cohort

GIRaFH (Genetic Identification of Risk factors in Familial Hypercholesterolemia) was a retrospective, multicenter (27 Dutch lipid clinics) study including 2400 unrelated heterozygous FH patients of Caucasian origin as previously described [26]. The mean follow-up period was 5 years. The primary outcome of the study was the combination of cardiovascular mortality and CVD as described [26]. Genomic DNA of 1994 FH patients was available for genotyping in the present study. The Ethics Institutional Review Board of each participating hospital approved the study.

Genetic analysis
In the ASAP trial, a 496-bp fragment of the *ANXA5* promoter (261 base pairs upstream and 235 base pairs downstream of the first transcription start point) was amplified by polymerase chain reaction (PCR) using two oligonucleotide primers: forward 5'CCGAGCCCTGGACAGCTCCCCA-3' and reverse 5'-GCCCCGCGACCACGCTCTCCTCT-3' as described [20]. Exon 2 with flanking regions (130-bp fragment) was amplified as previously described [27]. Purified amplicons were sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and analyzed on the ABI 3730 PRISM DNA Analyzer (Applied Biosystems). We evaluated six SNPs within the *ANXA5* promoter (rs62319820, rs112782763, rs28717001, rs28651243, rs113588187, rs1050606) and rs1131239 located in the Kozak sequence (exon 2) to reconstruct the four known haplotypes as described [23]. Successful sequencing of the promoter region and exon 2 was possible in 284 (95.0%) and 298 (99.7%) patients, respectively.

In the GIRaFH study, we genotyped four *ANXA5* SNPs (rs62319820, rs113588187, rs1050606, rs1131239) to reconstruct the four common *ANXA5* haplotypes. Genotyping was carried out using predesigned or custom TaqMan primers with FAM or VIC as fluorophores (Applied Biosystems, USA). SNP genotyping success rates were 93.5% (1864 patients) for rs62319820, 94.6% (1886 patients) for rs113588187, 92.8% (1850 patients) for rs1050606 and 95.9% for rs1131239 (1912 patients).

Biochemical measurements
Total cholesterol, (calculated) LDL-C, high-density lipoprotein cholesterol (HDL-C) and triglycerides were determined by standard methods as previously described [25;26]. High-sensitivity C-reactive protein (hs-CRP) was measured in citrated plasma by a commercially available enzyme-immunoassay (Dako, Denmark) as described [28]. Plasma ANXA5 levels were measured in 141 participants from the ASAP study using a commercially available Zymutest ANXA5 ELISA (Hyphen Biomed). The intra- and inter-assay coefficients of variation (CV) for ANXA5 measurements were 3.1% and 3.8% respectively.

Statistical analyses
The means of continuous variables of different groups were compared by unpaired t-test or one-way ANOVA as appropriate; the $\chi 2$ test was used for categorical variables. Hardy-Weinberg equilibrium for each SNP was evaluated by the $\chi 2$ test. Haploview software (Broad Institute, Cambridge, MA, USA) was used to estimate the degree of linkage disequilibrium (LD; r^2 values) between all SNP pairs and to determine haplotypes (H). Haplotypes were assigned manually to the subjects with complete genetic data as described [23]. Only common haplotypes (frequency >1%) were used for statistical analyses. In the present study, we included 284 FH patients from the ASAP study as well as 1730 FH patients from the GIRaFH cohort in whom common haplotypes were constructed. Power calculations were performed to determine the minimal odds ratio detectable with a power >80% (Quanto version 1.2.4 software).

In the ASAP study, the age-related IMT progression in different *ANXA5* haplotype groups was estimated by a linear regression analysis (SAS version 6.12 software). The interaction term (haplotype group x age) was entered in the regression models to account for different IMT progression rates with age between *ANXA5* haplotype groups. The variable gender associated with IMT in a univariable regression model was included in the multiple regression model. The regression coefficient β represents IMT increase with age (millimeters per year, mm/year). Comparison of the regression slopes between groups was performed by testing of the interaction terms. In the single SNP analyses, the slopes of the major allele carriers were taken as the reference. In the haplotype model, we performed one-way ANOVA for each haplotype to address the difference in regression slopes within the haplotype group. To control for the familywise error rate over the 4 haplotypes, the level of significance for each interaction test was set at 0.05/4=0.0125 (Bonferroni correction for multiple testing). When the p-value of the interaction test was less than 0.0125, further analysis with t-test was warranted to assess the difference between two specific slopes (e.g. carriers and non-carriers).

The distribution of plasma ANXA5 levels was normalized by ln-transformation and used in all analyses. A one-way ANOVA followed by the Bonferroni Post Hoc test was performed to assess differences in plasma ANXA5 levels within haplotype groups. The Bonferroni threshold for correction for multiple testing was estimated at 0.05/4=0.0125, taking into account the number of haplotypes (four). All values reported were reconverted to geometric means with the appropriate 95% confidence interval (CI). The association of plasma ANXA5 levels with carotid IMT was tested by a linear regression analysis and Pearson correlation. To test the relationship between circulating ANXA5 levels and total cholesterol, triglycerides, HDL-C, LDL-C and hs-CRP, we calculated Pearson's correlation coefficients. Skewed distributed variables (triglycerides and hs-CRP) were also ln-transformed prior to analysis.

In the GIRaFH study, the contribution of *ANXA5* variations to CVD risk was examined by Cox proportional hazards regression. The follow-up period started at birth and ended at the first occurrence of established fatal or non-fatal CVD event. Patients without CVD were censored at the date of the last lipid clinic visit or at the date of death attributable to other causes than CVD. In all analyses, we included year of birth, sex and smoking in the Cox regression models.

RESULTS

Patient characteristics of the ASAP study

Clinical characteristics of 284 FH patients participating in the ASAP study are shown in **Table 1**. Baseline characteristics of the study group were comparable to those of the total ASAP cohort (data not shown) [25]. The population consisted of Caucasian individuals with the mean age of 48.4 years; sixty percent of the subjects were female. Twenty-nine percent of the patients had a history of CVD, and 60.6% individuals were smokers (former and current).

Table 1. Baseline characteristics of FH patients from the ASAP study.

Characteristics	n=284
Age, years	48.4 ± 10.4
Gender, male/female, n (%)	114 (40.1)/170 (59.9)
History of CVD, n (%)	82 (28.9)
Smoking, n (%)†	172 (60.6)
Body mass index, kg/m^2	25.7 ± 3.5
Total cholesterol, mmol/L	10.13 ± 1.99
HDL-C, mmol/L	1.16 ± 0.31
LDL-C, mmol/L	8.17 ± 1.96
Triglycerides, mmol/L	1.64 (1.12-2.28)
Hs-CRP, mg/L	2.2 (0.8-4.6)
Carotid IMT, mm*	0.93 ± 0.22
CCA-IMT, mm*	0.87 ± 0.17

Values are presented as means±SD or n (%). Triglycerides and hs-CRP are given as median (interquartile range).
†Previous and current smokers.
*Data of 282 patients are shown.
CVD, cardiovascular disease; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; hs-CRP, high-sensitivity C-reactive protein; IMT, intima-media thickness; CCA-IMT, common carotid artery intima-media thickness; mm, millimeter.

Effect of plasma ANXA5 levels on progression of atherosclerosis in ASAP

We investigated the association between plasma ANXA5 levels and carotid IMT in a subset of subjects (n=141) randomly selected from the ASAP cohort. Plasma ANXA5 levels did not differ significantly between male (n=50) and female (n=91) subjects (geometric mean 13.17 µg/L, 95% CI: 11.01-15.75 versus 12.81 µg/L, 95% CI: 10.98-14.94, p=0.82) and were not influenced by age (linear regression: β=-0.009, p=0.10). Plasma ANXA5 levels did not correlate with cholesterol (total, HDL, LDL) levels, triglycerides levels and an inflammation marker hs-CRP (**Table S1**). No association was found between plasma ANXA5 and carotid IMT (linear regression: β=-0.01, p=0.8).

***ANXA5* SNPs and haplotypes in ASAP**

Table S2 shows the minor allele frequencies and the genotype frequencies of the seven SNPs in ANXA5 in the ASAP cohort. All SNPs were in Hardy-Weinberg equilibrium. Haploview analysis showed a high degree of linkage disequilibrium between all SNPs except for SNP1 and SNP6 (**Figure S1**). SNP3 and SNP4 as well as SNP2 and SNP5 were completely linked (r2=1). SNP7 was tightly linked to SNP2 and SNP5 (r^2=0.85). Based on the seven polymorphisms, the four previously reported common haplotypes were constructed (**Table 2**). Haplotype H1, the most frequent haplotype (51%), was composed of the major alleles of all seven polymorphisms. Haplotype H2 was discriminated from haplotype H1 by rs1050606 (SNP6). Haplotype H3 was the third major haplotype (9.9%) and consisted of the major allele of rs62319820 and the minor alleles of the other polymorphisms. Haplotype H4 compiled the minor alleles of rs62319820, rs28717001, rs28651243, rs1050606 and the major alleles of the three other SNPs. Besides the common haplotypes, three rare haplotypes (H5, H6, H7; frequency <1%) were identified in nine patients (**Table 2**). They were excluded for further analyses.

***ANXA5* haplotypes and plasma ANXA5 levels**

Among the ASAP participants, a one-way ANOVA corrected for multiple testing revealed significant differences in plasma ANXA5 levels within haplotypes H2 and H4 (**Table 3**). Haplotype H2 was associated with decreased plasma ANXA5 levels. Post Hoc analysis indicated that homozygous H2 carriers had lower ANXA5 levels (mean 6.20 µg/L, 95% CI: 4.24-9.05) compared to heterozygous subjects (mean 13.33 µg/L, 95% CI: 11.13-15.94, p<0.001) and non-H2 subjects (mean 14.25 µg/L, 95% CI: 12.18-16.69, p<0.001). There were no differences in plasma ANXA5 levels between H2 heterozygotes and non-H2 individuals (p=0.568).

Table 2. Polymorphisms and haplotypes of the *ANXA5* gene in 284 FH patients from the ASAP trial.

	SNP1	**SNP2**	**SNP3**	**SNP4**	**SNP5**	**SNP6**	**SNP7**	
	g.-628C>T	g.-467G>A	g.-448A>C	g.-422T>C	g.-373G>A	g.-302T>G	g.-1C>T	
	c.-390C>T	c.-229G>A	c.-210A>C	c.-184T>C	c.-135G>A	c.-64T>G	c.-1C>T	
dbSNP ID	rs62319820	rs112782763	rs28717001	rs28651243	rs113588187	rs1050606	rs1131239	
Haplotype								**Haplotype frequency**
H1	C	G	A	T	G	T	C	0.51
H2	C	G	A	T	G	**G**	C	0.29
H3	C	**A**	**C**	**C**	**A**	**G**	**T**	0.099
H4	**T**	G	**C**	**C**	G	**G**	C	0.09
H5	C	**A**	**C**	**C**	**A**	**G**	C	0.007
H6	C	G	A	T	G	**G**	**T**	0.005
H7	C	G	**C**	**C**	G	**G**	**T**	0.002

Nucleotide numbering from the ATG initiation codon; SNP, single nucleotide polymorphism;
dbSNP indicates NCBI database for SNPs (http://www.ncbi.nlm.nih.gov/snp); minor alleles in bold and underlined.

Furthermore, non-H4 individuals (mean 10.98 µg/L, 95% CI: 9.73-12.39) had a 2-fold lower circulating ANXA5 levels compared to homozygous H4 carriers (mean 29.67 µg/L, 95% CI: 15.53-44.44, p=0.007) and heterozygous H4 subjects (mean 24.48 µg/L, 95% CI: 19.99-30.02, p<0.001). Plasma ANXA5 levels were not different between homozygotes for H4 and heterozygotes for H4 (p=0.615).

With respect to the relationship of carotid IMT with lower plasma ANXA5 levels in H2 carriers as well as with higher plasma ANXA5 levels in H4 carriers, additional analyses did not reveal any statistical evidence for such association which might also be explained by the low number of cases in these subgroup analyses.

Table 3. Association of *ANXA5* haplotypes with plasma ANXA5 levels in FH patients from the ASAP study.

Haplotype	Number n=141	Geometric mean ANXA5 (95% CI), µg/L	Overall *p*-value
Haplotype 1			0.875
H1H1	36	13.42 (10.90-16.53)	
H1Hx	69	13.00 (11.19-15.09)	
Non-H1	36	12.33 (9.07-16.78)	
Haplotype 2			0.0005
H2H2	12	6.20 (4.24-9.05)	
H2Hx	56	13.33 (11.13-15.94)	
Non-H2	73	14.25 (12.18-16.69)	
Haplotype 3			0.018
H3Hx	30	9.89 (7.48-13.09)	
Non-H3	111	13.90 (12.24-15.77)	
Haplotype 4			<0.0001
H4H4	3	29.67 (15.53-44.44)	
H4Hx	25	24.48 (19.99-30.02)	
Non-H4	113	10.98 (9.73-12.39)	

Hx indicates all haplotypes except for the one given; CI, confidence interval. Differences were assessed by one-way ANOVA; the threshold for Bonferroni correction for multiple testing: 0.05/4 haplotype groups=0.0125

***ANXA5* haplotypes and progression of atherosclerosis in ASAP**
A linear regression analysis approach was applied to study the association between *ANXA5* variations and age-related carotid IMT (i.e., the average of available IMT values measured in three carotid segments). None of the *ANXA5* SNPs was associated with the age-related increase in carotid IMT (**Table S3**). Of the four haplotypes studied the *ANXA5* haplotype H3 tended to be associated with more rapid carotid IMT increase with age. Heterozygous haplotype H3 carriers tended to have a larger arterial wall thickening over time compared with non-H3 subjects, in both the unadjusted model (β=0.0129 mm/year versus β=0.0072 mm/year, p=0.053) and after adjustment for gender (β=0.0135 mm/year versus β=0.0076 mm/year, p=0.044) (Table 4). However, this association did not reach Bonferroni-corrected statistical significance level. Other *ANXA5* haplotypes did not demonstrate any association with the age-related IMT progression (**Table 4**).

Table 4. Association of *ANXA5* haplotypes with the age-related carotid IMT progression in FH patients from the ASAP trial.

	Unadjusted model		Adjusted model*	
Haplotype	**b (SE)**	**Overall *p*-value**	**b (SE)**	**Overall *p*-value**
Haplotype H1		0.27		0.30
H1H1 (n=77)	0.0062 (0.0024)		0.0066 (0.0023)	
H1Hx (n=128)	0.0077 (0.0017)		0.0083 (0.0017)	
Non-H1 (n=68)	0.0111 (0.0021)		0.0113 (0.0021)	
Haplotype H2		0.89		0.93
H2H2 (n=27)	0.0100 (0.0038)		0.0100 (0.0037)	
H2Hx (n=107)	0.0081 (0.0019)		0.0084 (0.0018)	
Non-H2(n=139)	0.0081 (0.0017)		0.0087 (0.0016)	
Haplotype H3		0.053		0.044
H3Hx (n=54)	0.0129 (0.0026)		0.0135 (0.0026)	
Non-H3 (n=219)	0.0072 (0.0013)		0.0076 (0.0013)	
Haplotype H4		0.48		0.55
H4H4 (n=4)	0.0153 (0.0067)		0.0153 (0.0066)	
H4Hx (n=41)	0.0067 (0.0027)		0.0075 (0.0026)	
Non-H4 (n=228)	0.0084 (0.0013)		0.0088 (0.0013)	

Results of the linear regression analysis (n=273) are shown. β indicates regression coefficient; β represents IMT increase with age (mm/year).*Adjusted for gender.
Hx means all haplotypes except for the one given; IMT, intima-media thickness; SE, standard error.

ANXA5 haplotypes and the risk of cardiovascular events

To confirm the results from the ASAP study, we investigated the contribution of ANXA5 variations to the risk of cardiovascular events in an independent cohort of 1730 FH patients from the GIRaFH study. Four *ANXA5* SNPs covering the four common *ANXA5* haplotypes were genotyped. All SNPs were in Hardy-Weinberg equilibrium (**Table S4**). Neither individual SNPs nor the common haplotypes were associated with CVD risk (**Table 5** and **Table S5**). No significant associations were found either with only homozygous wild type carriers (i.e., H1H1) as a reference category (data not shown). Subgroup analysis showed no significant associations between *ANXA5* variations and the history of myocardial infarction, angina pectoris, stroke/TIA or cardiac death (data not shown).

Table 5. Association of *ANXA5* haplotypes with CVD risk in FH patients from the GIRaFH study.

Haplotype	Number of subjects, n=1730	CVD+ (n=548) n (%)	HR (95% CI)*	*p*-value
Haplotype H1				
Non-H1	482	150 (27.4)	1.0	
H1Hx	883	278 (50.7)	0.9 (0.7-1.1)	0.5
H1H1	365	120 (21.9)	1.0 (0.8-1.3)	1.0
Haplotype H2				
Non-H2	777	257 (46.9)	1.0	
H2Hx	727	217 (39.6)	0.8 (0.7-1.0)	0.08
H2H2	226	74 (13.5)	1.0 (0.8-1.3)	0.9
Haplotype H3				
Non-H3	1358	424 (77.4)	1.0	
H3Hx	349	119 (21.7)	1.2 (1.0-1.5)	0.1
H3H3	23	5 (0.9)	0.8 (0.3-1.9)	0.6
Haplotype H4				
Non-H4	1463	465 (84.8)	1.0	
H4Hx	261	82 (15.0)	1.0 (0.8-1.3)	0.7
H4H4	6	1 (0.2)	NA	

CVD+, patients with cardiovascular disease; Hx, all haplotypes except for the one given; HR, hazard ratio; CI, confidence interval; NA, not applicable. HRs were calculated with HxHx (i.e., non-H1, non-H2, non-H3 and non-H4) as a reference category (HR=1.0).*Adjusted for sex, year of birth and smoking.

DISCUSSION

We investigated whether *ANXA5* gene variants and plasma ANXA5 levels were associated with atherosclerosis progression or CVD risk in FH patients. In our study, plasma ANXA5 levels were not associated with IMT. *ANXA5* haplotypes were associated with plasma ANXA5 levels, but did not correlate with carotid IMT parameters in the ASAP study or CVD risk in the GIRaFH cohort. Our data, therefore, showed a lack of association of ANXA5 protein levels as well as *ANXA5* haplotypes with carotid IMT progression or the risk of cardiovascular events in FH patients.

A possible role of ANXA5 in the pathophysiology of atherosclerosis has been postulated based on the observations that ANXA5 has anti-atherogenic and anti-inflammatory properties and is found in high concentrations in atherosclerotic plaques. Of note, it has been shown that the uptake of labeled recombinant ANXA5 by atherosclerotic plaques correlates with the extent of apoptosis [29]. In this regard, reduced plasma ANXA5 levels in patients with severe coronary stenosis have been proposed to reflect the presence and extent of CVD [24]. Moreover, in systemic lupus erythematosus (SLE) patients, antiphospholipid antibody-mediated reduced binding of ANXA5 to endothelial cells was found to be associated with IMT and it has been suggested to be an important mechanism in SLE-related CVD [3]. These observations prompted us to investigate whether ANXA5 plasma levels and *ANXA5* gene variants are associated with atherosclerosis progression in a high risk population of FH patients. The FH patient population was chosen because of its homogeneity, characterized by elevated LDL-C levels and an increased risk of premature CVD [30]. We hypothesized that *ANXA5* variants associated with circulating plasma ANXA5 levels would predict clinical features of atherosclerosis and CVD risk in FH patients.

The four promoter polymorphisms (rs112782763, rs28717001, rs28651243, rs113588187) evaluated in this study are known functional SNPs [20]. The rs112782763, which is located 19 nucleotides upstream from the transcription start site 1 (tsp1) in the gGCCc sequence, affects the zinc finger binding of the MTF-1 (metal-regulatory) transcription factor [31]. The substitution rs28717001 changes the tsp1 itself. The third polymorphism rs28651243 located 27 nucleotides downstream from the tsp1 disrupts a SP1 (specificity protein 1 transcription factor) consensus. The rs113588187 destroys a BamHI restriction site in the close proximity of an AP-4 (motif B)/MED-1 consensus, which in turn is essential for the full *ANXA5* promoter activity [20;32]. The *ANXA5* M2 haplotype (including our haplotype H3) comprising the four mentioned above promoter SNPs is related to a reduced *ANXA5* promoter activity in vitro [20] and reduced mRNA levels in placental tissues [33]. Altogether, it is thought that through reduced ANXA5 expression on the surface of placental trophoblasts and inefficient phospholipid shielding, this haplotype contributes to a prothrombotic placental environment [20]. In addition, SNP rs1131239 located in the *ANXA5* Kozak sequence was chosen since the minor rs1131239T-allele has previously been reported to

be associated with a protective role in the risk for myocardial infarction [16;17]. Furthermore, we also evaluated the rs62319820, because its minor T-allele is known to be a major contributor to higher plasma ANXA5 levels in healthy controls [23].

We initially found a trend towards a larger arterial wall thickening over time in heterozygous H3 patients compared to non-H3 carriers in the ASAP study, suggesting a possible clinical relevance of ANXA5 H3 in susceptibility to cardiovascular events. Remarkably, such a larger arterial wall thickness progression observed in *ANXA5* H3 carriers (13.5 μm/year after adjusting for sex) should contribute to a higher incidence of cardiovascular events, resulting in an increased CVD risk in these individuals. Unfortunately, no such effect was found while studying the CVD risk in a large population of FH patients from the GIRAFH study, which was designed to substantiate genetic risk factors in FH patients. Power calculations showed that the GIRaFH study had a ~85% power (α=0.05) to detect a clinically relevant odds ratio of 1.5 for *ANXA5* H3 (haplotype frequency of 0.10; an assumed prevalence of the disease of 30%). Since replication in a larger study including patients with a similar disease phenotype failed, we have to consider our initial finding, a trend for an association of ANXA5 H3 with IMT in the ASAP study, as a false-positive result or as a type I error.

Evidence for an association between *ANXA5* genetic variants and carotid IMT or CVD risk in atherosclerosis is scarce. Recently, it has been shown a moderate association between *ANXA5* intronic SNPs rs4833229, rs6830321 and the risk on restenosis in patients undergoing percutaneous coronary intervention for atherosclerosis (odds ratio 1.29, pallelic=0.011 and odds ratio 1.35, pallelic=0.003, respectively) [15]. A possible explanation for the discrepancy with this study could be explained by the pathophysiology underlying FH. Since atherosclerosis is a multifactorial disease, the contribution of genetic variations to CVD risk should be considered in the context of a chronic inflammatory disease of the arterial wall. It is known that elevated plasma LDL-C levels in FH patients maintain a chronic inflammatory environment within the arterial wall. The effects of LDL-C on the inflammatory reactions in atherosclerosis appear to be more dominant than the small effects of endogenous ANXA5. Our observation that plasma ANXA5 levels were not associated with cholesterol levels or an inflammation marker may support the minor impact of endogenous ANXA5 on inflammation in atherosclerosis. Of note, as our study was restricted to FH patients, the role of *ANXA5* genetic variations in atherosclerosis should be examined in other patient populations in order to understand its true physiological impact.

In conclusion, the data obtained from two independent cohorts of FH patients indicate that both common genetic variants in *ANXA5* and plasma ANXA5 levels are not associated with carotid IMT parameters or CVD risk.

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Conflict of interest
None declared.

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SUPPLEMENTAL DATA

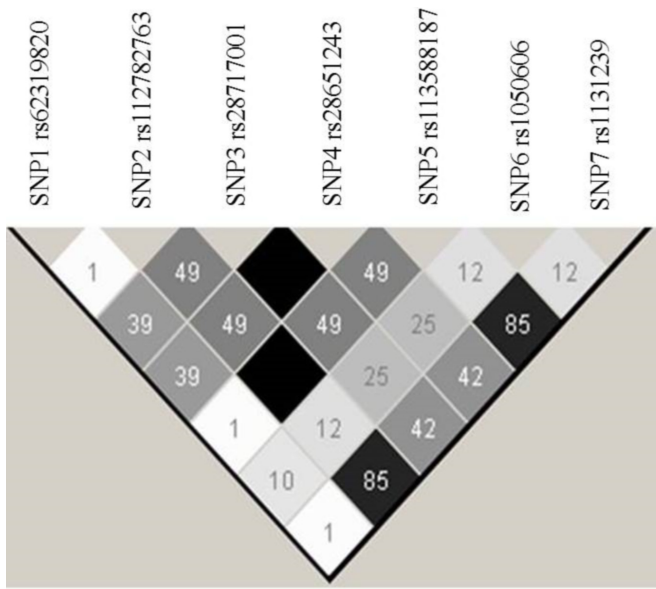


Figure S1. HAPLOVIEW LD plot of the seven SNPs within the promoter and 5'-untranslated region of the *ANXA5* gene. The LD coefficients r2 (x100) between all SNP pairs are shown in squares. The darker the gray color is, the higher the degree of LD. The black color (r^2=1) indicates complete linkage.

Table S1. Correlations of plasma ANXA5 levels with cholesterol, triglycerides, hsCRP levels and carotid IMT

		ANXA5, µg/L	
Variable	**Number**	***r***	***p*-value**
Total cholesterol, mmol/L	145	0.05	0.5
Triglycerides, mmol/L	145	0.03	0.7
HDL-C, mmol/L	145	0.06	0.9
LDL-C, mmol/L	144	0.03	0.7
Hs-CRP, mg/L	143	0.02	0.8
Carotid IMT, mm	145	-0.03	0.8

HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; hs-CRP. high-sensitivity C-reactive protein; IMT, intima-media thickness. r, Pearson correlation coefficient. Plasma ANXA5, triglycerides and hs-CRP levels were ln-transformed prior to analyses.

Table S2. Frequency distribution of *ANXA5* SNPs in FH patients from the ASAP trial and expected frequencies according to Hardy-Weinberg Equilibrium (n=284)

Polymorphism		MAF	Observed genotype	Expected genotype	*p*-value
SNP1	**rs62319820**	0.09			0.16
	CC		239	237	
	CT		41	45	
	TT		4	2	
SNP2	**rs112782763**	0.11			0.46
	GG		226	227	
	GA		56	54	
	AA		2	3	
SNP3	**rs28717001**	0.19			0.61
	AA		186	185	
	AC		86	88	
	CC		12	11	
SNP4	**rs28651243**	0.19			0.61
	TT		186	185	
	TC		86	88	
	CC		12	11	
SNP5	**rs113588187**	0.11			0.46
	GG		226	227	
	GA		56	54	
	AA		2	3	
SNP6	**rs1050606**	0.49			0.29
	TT		79	75	
	TG		133	141	
	GG		72	68	
SNP7	**rs1131239**	0.10			0.19
	CC		226	228	
	CT		57	53	
	TT		1	3	

MAF, minor allele frequency.

Table S3. Association of individual *ANXA5* SNPs with age-related carotid IMT in FH patients from the ASAP trial

	Unadjusted model		Adjusted model*	
SNP/genotype	**β**	***p*-value**	**β**	***p*-value**
SNP1 rs62319820				
CC (n=237)	0.0087	Ref.	0.0093	Ref.
CT+TT (n=45)	0.0084	0.93	0.0091	0.94
SNP2 rs112782763†				
GG (n=226)	0.0076	Ref.	0.0081	Ref.
GA (n=56)	0.0130	0.059	0.0136	0.05
SNP3 rs28717001‡				
AA (n=184)	0.0074	Ref.	0.0078	Ref.
AC+CC (n=98)	0.0105	0.17	0.0111	0.13
SNP6 rs1050606				
TT (n=78)	0.0066	Ref.	0.0070	Ref.
TG+GG (n=204)	0.0093	0.31	0.0098	0.28
SNP7 rs1131239				
CC (n=225)	0.0076	Ref.	0.0081	Ref.
CT (n=57)	0.0126	0.07	0.0132	0.06

Results of the linear regression analysis (n=282) are shown.
β indicates regression coefficient; **β** represents IMT increase with age (mm/year)
The slopes of the major allele carriers were taken as the reference.
*Adjusted for gender and study center.
†The same results for SNP5 rs113588187 due to complete linkage
‡The same results for SNP4 rs28651243 due to complete linkage
IMT means intima-media thickness; Ref., reference.

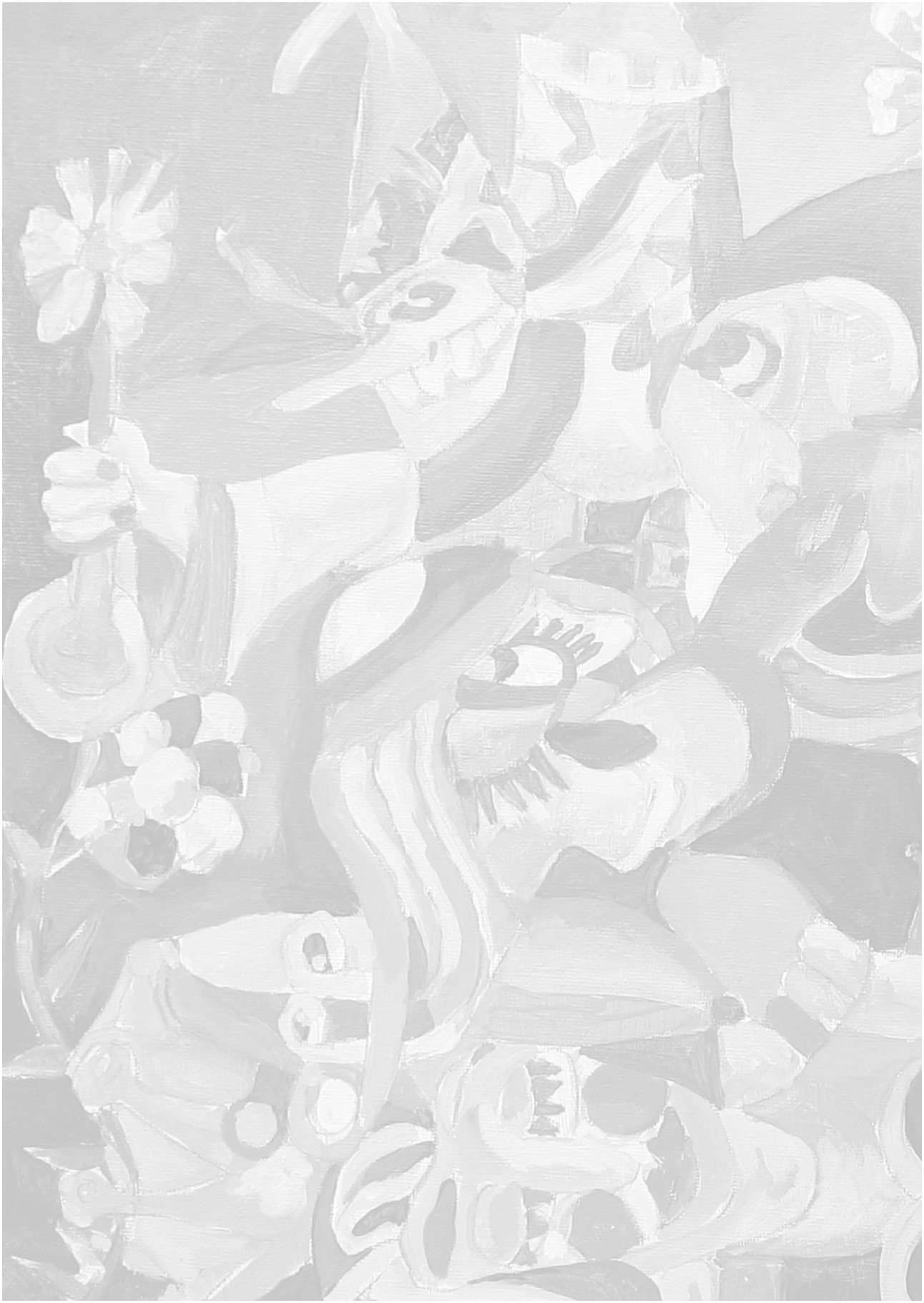
Table S4. Frequency distribution of *ANXA5* SNPs in FH patients from the GIRaFH study and expected frequencies according to Hardy-Weinberg Equilibrium

Polymorphism	Observed genotype	Expected genotype	p-value
rs62319820	**n=1864**		0.99
CC	1534	1534	
CT	314	314	
TT	16	16	
rs113588187	**n=1886**		0.81
GG	1478	1477	
GA	382	384	
AA	26	25	
rs1050606	**n=1850**		0.77
TT	415	418	
TG	929	923	
GG	506	509	
rs1131239	**n=1912**		0.88
CC	1494	1495	
CT	393	392	
TT	25	26	

Table S5. Association of ANXA5 polymorphisms with CVD risk in FH patients from the GIRaFH study.

Polymorphism	CVD- n (%)	CVD+ n (%)	HR (95% CI)*	*p*-value
rs62319820	**n=1279**	**n= 585**		
CC	1047 (81.9)	487 (83.2)	1.0	
CT	218 (17.0)	96 (16.4)	1.0 (0.8-1.2)	0.7
TT	14 (1.1)	2 (0.3)	0.3 (0.04-2.0)	0.2
rs113588187	**n= 1295**	**n=591**		
GG	1019 (78.7)	459 (77.7)	1.0	
GA	256 (19.8)	126 (21.3)	1.1 (0.9-1.4)	0.2
AA	20 (1.5)	6 (1.0)	0.8 (0.4-1.9)	0.7
rs1050606	**n=1269**	**n=581**		
TT	282 (22.2)	133 (22.9)	1.0	
TG	635 (50.0)	294 (50.6)	0.9 (0.8-1.2)	0.6
GG	352 (27.7)	154 (26.5)	1.0 (0.8-1.3)	1.0
rs1131239	**n=1314**	**n=598**		
CC	1032 (78.5)	462 (77.3)	1.0	
CT	262 (19.9)	131 (21.9)	1.2 (0.9-1.4)	0.2
TT	20 (1.5)	5 (0.8)	0.7 (0.3-1.6)	0.4

CVD+, patients with cardiovascular disease; CVD-, patients without cardiovascular disease;
HR, hazard ratio; CI, confidence interval.
HRs were calculated with the major allele as a reference category (HR=1.0).
*Adjusted for sex, year of birth and smoking.



Chapter 5

No association between *Annexin A5* genetic variants and deep venous thrombosis

Paulien G. de Jong*, Larissa Hiddink*, Joost C.M. Meijers, Lambertus A. Kiemeney, Waander L. van Heerde, and Saskia Middeldorp

***Both authors contributed equally**

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ABSTRACT

Introduction: Annexin A5 (ANXA5) is a protein with antithrombotic properties present in vascular endothelium. It has been suggested that genetic variants in the *Annexin A5* (*ANXA5*) gene affect ANXA5 expression, contributing to a local procoagulant state. Some studies showed associations of *ANXA5* single nucleotide polymorphisms (SNPs) or haplotypes with pregnancy-related deep venous thrombosis (DVT) and myocardial infarction.
Objectives: To investigate whether common variants in the *ANXA5* promoter are associated with DVT risk in Dutch Caucasian individuals.
Methods and Results: From the Amsterdam Case-control Thrombophilia (ACT) study, 148 patients with newly diagnosed DVT and 267 controls without previous VTE were included. We sequenced the promoter region of the *ANXA5* gene and reconstructed four common haplotypes, based on six SNPs. Neither individual SNPs nor any of the common haplotypes were associated with an increased risk for DVT. Furthermore, the four *ANXA5* haplotypes were equally distributed among DVT patients and a second independent control group of 1705 individuals from the general population (Nijmegen Biomedical Study).
Conclusions: Our data suggest that *ANXA5* haplotypes do not contribute to DVT risk in the Dutch population.

INTRODUCTION

Venous thromboembolism (VTE) is a multifactorial disease with an incidence of 1-2 per 1000 per year in western countries [1]. Although several genetic risk factors for VTE such as factor V Leiden or prothrombin G20210A mutation have been identified, the risk of VTE is also increased in case of a positive family history of VTE, in whom known genetic risk factors have been ruled out [2]. This suggests that other as yet unknown genetic variants also predispose to VTE.

Annexin A5 (ANXA5) is a natural anticoagulant protein highly expressed by vascular endothelium and placental trophoblasts [3-5]. The hypothesis of a possible role of ANXA5 in the pathogenesis of thrombotic disorders originates from its anticoagulant, antithrombotic and anti-inflammatory properties observed in vitro and in animal models in vivo [6-9]. In the presence of Ca^{2+}-ions, ANXA5 binds and shields anionic phospholipids, which form the catalytic surface for coagulation reactions [10;11]. Reduced ANXA5 expression on the surface of vascular cells and subsequently inefficient shielding of anionic phospholipids could contribute to the activation of blood coagulation and the creation of a prothrombotic environment within the blood vessel.

In the antiphospholipid syndrome, for example, a reduction of ANXA5 at the vascular wall is thought to be one of the several explanations for the occurrence of both arterial and venous thrombosis [12-14]. Reduced binding of ANXA5 to cardiolipin observed in patients with confirmed idiopathic venous thrombosis, supports the role of ANXA5 in thrombosis [15]. In patients with systemic lupus erythematosus, reduced binding of ANXA5 to endothelium has also been proposed as a mechanism underlying atherothrombosis [16]. In addition, ANXA5 down-regulates expression of the procoagulant tissue factor, which is a key player in VTE [9;17;18]. Furthermore, ANXA5 is known to be an effective inhibitor of experimentally induced venous and arterial thrombosis in animal models [8;19].

If genetic variants within the *ANXA5* gene affect ANXA5 expression on cell surfaces, these could also influence the risk of clinical outcomes such as arterial or venous thrombosis. Several studies have been performed to assess this. A genetic variant located in the Kozak sequence (g.-1C>T, rs1131239) of the *ANXA5* gene was associated with higher plasma ANXA5 levels [20]. This minor rs1131239T-allele was associated with a decreased risk of myocardial infarction in young patients [20] and a lower risk of developing a new coronary event during 36 months follow-up [21]. Other studies, however, were unable to reproduce these findings [22;23]. In the study that showed a decreased risk of myocardial infarction, venous thrombotic risk was also investigated but no significant association between the minor rs1131239T-allele and deep venous thrombosis (DVT) was found (OR for DVT 0.76, 95% CI 0.47-1.22) [20]. Similarly, in a Dutch population of 198 patients with autoimmune diseases, no association with venous or arterial thrombosis was found [24]. A haplotype comprising four *ANXA5* promoter single nucleotide polymorphisms (SNPs) (rs112782763, rs28717001, rs28651243, rs113588187), collectively referred to as the M2 haplotype, reduces *ANXA5* promoter activity in a promoter construct assay in vitro [25], which could be translated to an increased DVT risk. Indeed,

the presence of the M2 allele was found to be a risk factor for DVT in pregnancy or the postpartum period [26] as well as in the general Southern Italian population [27].
In a previous study, we described four common *ANXA5* haplotypes (H1, H2, H3 and H4) [28]. Of these, the haplotype H3 is an extension of the previously described M2 haplotype that reduces *ANXA5* promoter activity in vitro [25] as well as correlates with lower plasma ANXA5 levels [28]. Interestingly, the minor rs1131239T-allele, which was associated with increased plasma ANXA5 levels and a decreased risk of myocardial infarction [20], is completely linked to SNPs comprising the M2 haplotype [28].
Given the conflicting results on the association of variants within the *ANXA5* gene upstream region with clinical outcomes in previous studies, we aimed to evaluate whether *ANXA5* promoter SNPs and haplotypes influence the risk of DVT in the Dutch general population. For this study, we used a case-control study on risk factors for DVT, the Amsterdam Case-control Thrombophilia (ACT) study, and a second group of population controls (Nijmegen Biomedical Study, NBS).

MATERIALS AND METHODS

Study population

From the Amsterdam Case-control Thrombophilia Study (ACT) performed between September 1999 and May 2006 [29], we selected 437 unrelated individuals (154 cases and 283 controls) of Caucasian origin. Both cases and controls were from the Amsterdam region, the western part of the Netherlands. Cases were patients with newly diagnosed and objectively confirmed proximal DVT of the leg. Controls were patients without previous VTE, in whom DVT was suspected but ruled out. The diagnosis of DVT was based on the Wells score and D-dimer plasma level algorithm, followed by compression ultrasonography if indicated, as described [29;30].
A standardised questionnaire was used for all participants, to obtain information about known risk factors such as malignancy or treatment because of malignancy in the last 6 months; pregnancy or postpartum period; use of oral contraceptives or hormonal therapy; trauma within the last 60 days; being bedridden (for >3 days); paralysis or recent plaster immobilisation of the symptomatic leg; surgery within the last 4 weeks. Information was obtained prior to diagnosis of DVT, i.e. classification as case or control. Genomic DNA was isolated from peripheral leukocytes and was stored at +4°C. The Medical Ethical Committee of the Academic Medical Center in Amsterdam approved the study.
The second control group consisted of individuals who had been included in the Nijmegen Biomedical Study (NBS), of which details were reported previously [31]. Briefly, the NBS is a population-based survey conducted by the Department for Health Evidence and the Department of Laboratory Medicine of the Radboudumc. 21,756 age- and sex-stratified randomly selected inhabitants of the municipality of Nijmegen in the eastern part of the Netherlands received an invitation to fill out a postal questionnaire on, e.g., lifestyle and medical history, and to donate blood samples. The response to the questionnaire was 43% (N=9350). 69% (N=6468) of the responders donated blood samples. Of the 1819 cancer free NBS

participants who served as controls in a genome-wide association study [32], we selected for this study 1705 controls of self-reported European descent who reported not to have had DVT or pulmonary embolism.
Written informed consent was obtained from all ACT and NBS participants.

Genetic analysis
In the ACT subjects, a 496-bp fragment of the *ANXA5* promoter (261 base pairs upstream and 235 base pairs downstream of the first transcription start point) was amplified by polymerase chain reaction (PCR) using two oligonucleotide primers: forward 5' CCGAGCCCTGGACAGCTCCCCA-3' and reverse 5'-GCCCCGCGACCACGCTCTCCTCT-3' [25]. PCR reactions were carried out in a final volume of 25 µl reaction mixture containing 2.5 µl 10x PCR Buffer (Qiagen), 5% DMSO (v/v), 1 M Betaine, 0.4 µM of each primer (forward and reverse), 0.08 mM of each deoxynucleotide triphosphate, 100 150 ng genomic DNA and 1.25 U Taq DNA polymerase (Qiagen). Cycling conditions were: an initial denaturation step at 95°C for 3 minutes followed by denaturation at 95°C for 1 minute, annealing at 62°C for 1 minute and elongation at 72°C for 1 minute (30 cycles in total). PCRs were performed in a T3 Thermal Cycler (Biometra, Germany). Sequence analysis was performed by direct sequencing using the Big Dye Terminator ABI Prism Kit, version 1.1 (Applied Biosystems, Foster City, CA). Products of sequence reactions were analysed on a Genetic Analyzer 3730 (Applied Biosystems, Foster City, CA). Sequencing chromatograms were examined by the use of the Sequencer package (GeneCodes Co, Ann Arbor, MI).
NBS controls were genotyped using the Illumina HumanHapCNV370-Duo BeadChip as described [32]. For this study, we extracted 4 SNPs in *ANXA5* (rs62319820, rs113588187, rs1050606 and rs1131239) from genome-wide imputed SNP data using the "Genome of the Netherlands" (GoNL) data as reference.

Statistical analysis
Hardy-Weinberg equilibrium for each SNP was tested using the $\chi 2$ test. HAPLOVIEW software (Broad Institute, Cambridge, MA, USA) was used to estimate the degree of linkage disequilibrium (LD; r2 values) between all SNP pairs and to determine haplotypes (H) [33]. In the ACT participants, haplotypes were constructed using six promoter SNPs (rs62319820, rs112782763, rs28717001, rs28651243, rs113588187, rs1050606). As the minor rs1131239T-allele is completely linked to rs112782763 and rs113588187, haplotypes were equal to previously described elsewhere [28]. To reconstruct the four known *ANXA5* haplotypes in the NBS controls, we used three haplotype-tagging SNPs (rs62319820, rs113588187, rs1131239) and SNP rs1050606, of which the major T-allele is specific for haplotype H1. Haplotypes were assigned manually to all individuals.
The association of *ANXA5* SNPs and haplotypes with DVT risk was examined using the $\chi 2$ test. Odds ratios (ORs) with 95% confidence intervals (CI) were calculated as an estimate of the relative risk indicating the risk for DVT in a category of exposure (e.g., haplotype Hx carriers) relative to the reference category (e.g., non-haplotype Hx). ORs for DVT adjusted for age, sex, and presence of provoking risk factors were calculated using logistic regression. Statistical analyses were performed using SPSS version 20.0 software. Two-sided probability values of <0.05 were considered statistically significant.

RESULTS

Clinical characteristics of DVT patients and controls from the ACT study

In total, 148 DVT patients and 267 controls were included in the analyses, as sequencing of the *ANXA5* promoter was not successful in 6 patients and 16 controls. The characteristics of the study participants are presented in **Table 1**. The mean age was comparable between both groups, and patients were more often male than controls (p=0.046). Body mass index was similar in cases and controls (26.0 and 27.2 kg/m2 respectively). Thirty DVT patients (20.3%) had a prior history of VTE. In 77 patients (52%), risk factors for DVT were identified. Factor V Leiden was found in 23.1% of cases and in 5.8% of controls and the prothrombin G20210A mutation - in 4.1% cases and 2.9% controls.

Table 1. Characteristics of the study population.

	Case (n=148)	ACT controls (n=267)	NBS controls (n=1705)
Age, years	59.1 (16.0)	58.5 (16.7)	60.7 (10.3)
Male sex, n (%)	76 (51.4)	110 (41.2)	833 (48.9)
Provoking risk factors for VTE, *n* (%)†	77 (52.0)	107 (40.1)	N.A.
Factor V Leiden, *n* (%)*	34 (23.1)	14 (5.8)	90 (5.4)
Prothrombin G20210A mutation, *n* (%)¥	6 (4.1)	7 (2.9)	9 (0.54)
Previous VTE, *n* (%)	30 (20.3)	N.A.	N.A.

Continuous variables are presented as mean (standard deviation), categorical data as count (%).
VTE, venous thromboembolism; NA, not applicable
† malignancy or treatment because of malignancy in the last 6 months; pregnancy or postpartum period; use of oral contraceptives or hormonal therapy; trauma within the last 60 days; being bedridden (for > 3 days); paralysis or recent plaster immobilisation of the symptomatic leg; surgery within the last 4 weeks
*Data from 147 cases and 242 ACT controls (including 1 homozygous case and 1 homozygous control) and 1678 NBS controls (including 1 homozygous)
¥ Data from 147 cases and 241 ACT controls (including 1 homozygous control) and 1676 NBS controls.

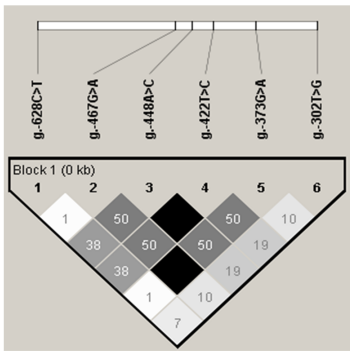
ANXA5 SNPs and haplotypes in the ACT study

Sequencing of the *ANXA5* promoter showed the presence of six common polymorphisms (**Figure 1**), i.e. SNP1 (rs62319820, g.-628C>T), SNP2 (rs112782763, g.-467G>A), SNP3 (rs28717001, g.-448A>C), SNP4 (rs28651243, g.-422T>C), SNP5 (rs113588187, g.-373G>A) and SNP6 (rs1050606, g.-302T>G) that have previously been reported [25;28;34]. We also identified three rare variants. SNP7 (g.-622G>C) located six nucleotides downstream of SNP1 was found in three controls and one

A.

SNP	dbSNP ID	Risk allele frequencies		
		Patients (n = 148)	ACT controls (n=267)	NBS controls (n=1705)
SNP1 g.-628 C>**T**	rs62319820	0.08	0.09	0.08
SNP2 g.-467G>**A**	rs112782763	0.10	0.12	N.A.
SNP3 g.-448A>**C**	rs28717001	0.18	0.21	N.A.
SNP4 g.-422T>**C**	rs28651243	0.18	0.21	N.A.
SNP5 g.-373G>**A**	rs113588187	0.10	0.12	0.11
SNP6 g.-302T>**G**	rs1050606	0.52	0.57	0.51
SNP7 g.-622G>**C**	N.R.	0.01	0.006	N.A.
SNP8 g.-585G>**A**	N.R.	-	0.004	N.A.
SNP9 g.-506G>**A**	N.R.	-	0.004	N.A.
SNP10 g.-1C>**T**	rs1131239	not done	not done	0.11

B.



C.

Haplotype	Bogdanova's haplotype	
H1	N	CGATGT
H2	N	CGATGG
H3	M2	CACCAG
H4	M1	TGCCGG

Figure 1. *ANXA5* polymorphisms and haplotypes. (A) rs numbers and risk allele frequencies in patients and controls. Nucleotide numbering from the ATG initiation codon; SNP, single nucleotide polymorphism; dbSNP indicates NCBI database for SNPs http://www.ncbi.nlm.nih.gov/snp); risk alleles in bold and underlined; N.A., not applicable; N.R., not reported. Genotype frequencies were in Hardy Weinberg equilibrium both in ACT controls (SNPs 1-6) and in NBS controls (SNPs 1,5,6 and 10). (B) HAPLOVIEW Linkage Disequilibrium plot of the six SNPs within the *ANXA5* promoter in the ACT study. The LD coefficients r2 (x100) between all SNP pairs are shown in squares. The black colour (r2=1) indicates complete linkage. (C) Calling of haplotypes according to previous studies (25;28).

patient in a heterozygous form as well as in one patient in a homozygous form (MAF in controls: 0.006; MAF in patients: 0.01). SNP8 (g.-585G>A) and SNP9 (g.-506G>A) upstream of SNP2 were present in two controls in a heterozygous form (MAF: 0.004). These rare polymorphisms were excluded from further analyses.

The genotype frequencies of the common *ANXA5* SNPs were in Hardy-Weinberg equilibrium both in DVT patients and in controls (**Table S1**). We examined an association between separate SNPs and DVT risk (**Table S2**). None of the six polymorphisms was significantly associated with DVT.
Haploview analysis revealed a high degree of linkage disequilibrium between all SNPs except for SNP1 and SNP6. SNP2 and SNP5 as well as SNP3 and SNP4 were completely linked (r^2=1) (**Figure 1**). Only four common haplotypes (frequency > 1%) were present, which was similar to our previous study in a different Dutch population [28].

Table 2 shows the association between *ANXA5* haplotypes and DVT. Carriers of only major alleles for all SNPs (haplotypes H1, H1H1+H1Hx) appeared to have a slightly increased risk for DVT compared to non-H1 carriers, in both the unadjusted model (OR 1.5, 95% CI: 0.9-2.3) and after adjusting for age, sex and the presence of provoking risk factors (OR 1.4, 95% CI: 0.9-2.2), but the risk estimates did not reach statistical significance. The H2, H3 and H4 *ANXA5* haplotypes were not associated with DVT. Additionally, when homozygous carriers of H1 (H1H1) were used as the reference category instead of HxHx, the haplotypes H2, H3 and H4 were not associated with DVT (data not shown). Furthermore, we compared H3 carriers (encompassing the M2 haplotype, 27 cases and 59 controls) to carriers of only H1 and/or H2 (i.e., H1H1, H1H2, H2H2 genotypes; N/N in previous studies, 99 cases and 171 controls) and no association between H3 and DVT was found (OR 0.79, 95% CI 0.47 - 1.33). We also examined DVT risk for men and women separately. In men, haplotype H2-carriers (H2H2+H2Hx) appeared to have a slightly decreased risk of DVT compared to non-H2 carriers (OR 0.6, 95% CI: 0.3-1.1). Women carrying haplotype H3 tended to have a reduced risk for DVT (OR 0.5, 95% CI: 0.3-1.2). Again, these estimates did not reach statistical significance. Finally, subgroup analysis in 71 patients with unprovoked DVT did not also detect any association between *ANXA5* haplotypes and DVT (data not shown).

***ANXA5* SNPs and haplotypes in population controls**
To verify if the prevalence of *ANXA5* haplotypes in the selected hospital controls is representative of the population prevalence, we included an independent control group from the general population. Of the 1705 NBS controls, 48.9% was male and the mean age was 60.7 years (**Table 1**). The prevalence of Factor V Leiden in NBS controls (5.4%) was similar to that in ACT controls (5.8%), whereas carriership of the prothrombin G20210A mutation was less prevalent in NBS controls (0.54%) compared to ACT controls (2.9%).
Four *ANXA5* SNPs covering the four common *ANXA5* haplotypes were evaluated. The genotype distributions of all SNPs were in Hardy-Weinberg equilibrium (Table S3). The common *ANXA5* haplotypes were constructed in 1704 NBS controls as one control person presented with the rare *ANXA5* haplotype. Finally, all *ANXA5* haplotypes were similarly distributed among DVT patients and NBS controls (**Table 2**) as well as among subgroups of men or women (data not shown).

Table 2. Association of *ANXA5* haplotypes with DVT.

Haplotype	Patients n=148, n (%)	ACT Controls n=267, n (%)	OR for DVT (95% CI)	NBS Controls* n=1704, n (%)	OR for DVT (95% CI)
H1 (CGATGT)					
Non-H1	39 (26.4)	92 (34.5)	1	436 (25.6)	1
H1Hx	77(52.0)	119 (44.6)	1.5 (1.0 – 2.4)	875 (51.3)	1.0 (0.7 – 1.5)
H1H1	32 (21.6)	56 (21.0)	1.3 (0.8 – 1.4)	393 (23.1)	0.9 (0.6 – 1.5)
H1H1+H1Hx	109 (73.6)	175 (65.5)	1.5(0.9 – 2.3)	1268 (74.4)	1.0 (0.7 – 1.4)
Frequency H1	0.48	0.43		0.49	
H2 (CGATGG)					
Non-H2	66 (44.6)	112 (41.9)	1	775 (45.5)	1
H2Hx	61 (41.2)	116 (43.4)	0.9 (0.6 – 1.4)	752 (44.1)	1.0 (0.7 – 1.4)
H2H2	21 (14.2)	38 (14.2)	0.9 (0.5 – 1.7)	177 (10.4)	1.4 (0.8 – 2.3)
H2H2+H2Hx	82 (55.4)	154 (57.7)	0.9 (0.6 – 1.4)	929 (54.5)	1.0 (0.7 – 1.5)
Frequency H2	0.35	0.36		0.33	
H3 (CACCAG)					
Non-H3	121 (81.8)	208 (77.9)	1	1352 (79.3)	1
H3Hx	26 (17.6)	55 (20.6)	0.8 (0.5 – 1.4)	331 (19.4)	0.9 (0.6 – 1.4)
H3H3	1 (0.7)	4 (1.5)	0.4 (0.04 – 3.9)	21 (1.2)	0.5 (0.07 – 4.0)
H3H3+H3Hx	27 (18.2)	59 (22.1)	0.8 (0.5 – 1.3)	352 (20.7)	0.9 (0.6 – 1.3)
Frequency H3	0.09	0.12		0.11	
H4 (TGCCGG)					
Non-H4	125 (84.5)	221 (82.8)	1	1445 (84.8)	1
H4Hx	22 (14.9)	45 (16.9)	0.9 (0.5 – 1.5)	251 (14.7)	1.0 (0.6 – 1.6)
H4H4	1 (0.7)	1 (0.4)	1.8 (0.1 – 28.5)	8 (0.5)	1.4 (0.2 – 11.6)
H4H4+H4Hx	23 (15.5)	46 (17.2)	0.9 (0.5 – 1.5)	259 (15.2)	1.0 (0.6 – 1.6)
Frequency H4	0.08	0.09		0.08	

* One person carrying the rare haplotype was excluded from analysis.
All haplotypes but the one given (e.g. non-H1, non-H2, non-H3 or non-H4) were used as the reference category (OR = 1).
DVT, deep venous thrombosis

DISCUSSION

In this study, neither individual SNPs nor any of the four haplotypes within the *ANXA5* gene upstream region (including the H3 haplotype which is an extension of the M2 haplotype) were associated with an increased risk of DVT.

How do our findings relate to previous studies? First, there is a discrepancy in calling of haplotypes. Previous studies reported the M2 haplotype (comprising SNPs 2, 3, 4 and 5). We previously reported four other haplotypes (H1, H2, H3 and H4), of which H3 is an extension of the M2 haplotype (additionally including SNP6 and SNP7, rs1131239 g.-1C>T [28]). The studies that found an association between *ANXA5* haplotypes and DVT compared M2 carriers to non-M2 carriers [26;27]. According to our results, the non-M2 carriers include haplotypes H1 and H2 [28]. We found no association between haplotype H3 (the extension of the M2 haplotype) and DVT, neither when comparing H3 carriers to non-H3 carriers, nor when comparing H3-carriers to only H1 and/or H2 carriers (N/N in previous studies). It should be noted that the haplotypes H1 and H2 are associated with different plasma ANXA5 levels in healthy controls [28]. Although this suggests that H1 and H2 should not be combined as one reference category, data regarding the relevance of plasma ANXA5 levels in DVT patients are lacking. Nevertheless, this differentiation of haplotypes within the non-M2 control group could be of clinical importance. For example in other disease states, such as in recurrent pregnancy loss, plasma ANXA5 levels are known to be related to clinical features [35]. This subject therefore requires further investigation.

Second, one of the previous studies reporting an association of the *ANXA5* M2 haplotype with VTE was limited to pregnant women and to women in the postpartum period [26]. As only four of our patients had a DVT related to pregnancy, we could not investigate a possible association between *ANXA5* H3 and pregnancy-related thrombotic complications.
Third, the discrepancy with the Italian studies may be explained by differences between populations as we found a higher percentage of controls carrying *ANXA5* H3 (22.1% in ACT controls and 20.7% in NBS controls versus 14.4% and 16.6%, reported as the M2 haplotype in the Italian studies) (26;27). In our previous report, we showed a similar percentage of H3 carriers (21.4%) among healthy Dutch controls [28]. A higher percentage of healthy control subjects carrying the rs1131239T-allele (23.1%) was also found in the Spanish study [20]. As the frequency of SNPs varies for different ethnic groups, our study population was limited to Caucasian individuals.

It is possible that the lack of association we observed is due to a relatively small sample size of the ACT study. However, considering the genotype frequency of the H3 haplotype among controls of 22%, the study had a ~85% power (α=0.05) to detect an OR of 2.0 or more for DVT. Furthermore, with the use of a large cohort of population controls, the study was powered (~80%, α=0.05) to detect a clinically relevant OR of 1.7 for *ANXA5* H3 (genotype frequency of 20.6%).

We are aware that with the current study design, we cannot rule out the possibility that other genetic variants in regulatory regions of the *ANXA5* gene or other genes may be associated with DVT. This study, however, addressed the conflicting results in the literature and therefore analyses were restricted to the common *ANXA5* variants in light of the common disease – common variant hypothesis. In the search for new candidate genes that may be a risk factor for DVT, other strategies may be considered to be more suitable, thereby studying rare variants as opposed to common polymorphisms. In this respect, a next-generation DNA sequencing platform was applied for sequencing of the coding regions of 186 haemostatic/proinflammatory genes, including the *ANXA5* gene, in Italian patients with idiopathic DVT [36]. Interestingly, the intronic *ANXA5* polymorphism rs2306416 (g.A>G), which is located 57-bp downstream from the ATG codon, belonged to the top-5 variants selected for replication but failed to show any association with DVT after replication. Other *ANXA5* variants located in exons and exon-intron boundaries were not associated with DVT in an initial stage. This suggests that even with a different study design, investigating the coding regions of *ANXA5* besides the *ANXA5* promoter, no association between *ANXA5* SNPs and thrombosis risk will be found.

Despite the lack of association between *ANXA5* genetic variants and DVT, previous studies have shown that *ANXA5* genetic variants may play a role in the occurrence of obstetric complications such as miscarriage and intra-uterine growth restriction [25;34;37]. Furthermore, placental ANXA5 was shown to be critical for maintaining murine placental integrity [38] and in humans, reduced plasma ANXA5 levels were associated with recurrent pregnancy loss [35]. These associations require further research.
In conclusion, we could not demonstrate a meaningful association between *ANXA5* genetic variants and DVT in the Dutch population.

Addendum
The Amsterdam Case-control Thrombophilia Study was performed between September 1999 and May 2006 and DNA samples and clinical data of participants are stored at the Academic Medical Center, Amsterdam.

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Conflict of interest
The authors state that they have no conflict of interest.

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SUPPLEMENTAL DATA

Table S1. Frequency distribution of *ANXA5* polymorphisms and expected frequencies according to Hardy-Weinberg Equilibrium in the ACT study (DVT patients (n=148) and controls (n=267)).

Polymorphism	DVT patients		Controls	
	Observed	Expected†	Observed	Expected†
	n	*n*	*n*	*n*
SNP1 -628 C>T (rs62319820)				
CC	125	125	221	222
CT	22	22	45	43
TT	1	1	1	2
SNP2 -467 G>A (rs112782763)				
GG	121	121	208	208
GA	26	25	55	56
AA	1	1	4	4
SNP3 -448 A>C (rs28717001)				
AA	99	101	171	168
AC	46	43	82	87
CC	3	5	14	11
SNP4 -422 T>C (rs28651243)				
TT	99	101	171	168
TC	46	43	82	87
CC	3	5	14	11
SNP5 -373 G>A (rs113588187)				
GG	121	121	208	208
GA	26	25	55	56
AA	1	1	4	4
SNP6 -302 T>G (rs1050606)				
TT	32	34	56	50.
TG	77	74	119	131
GG	39	41	92	86

†Expected frequencies of all *ANXA5* SNPs were not statistically different from observed frequencies (p-value > 0.05), both in controls and in cases.

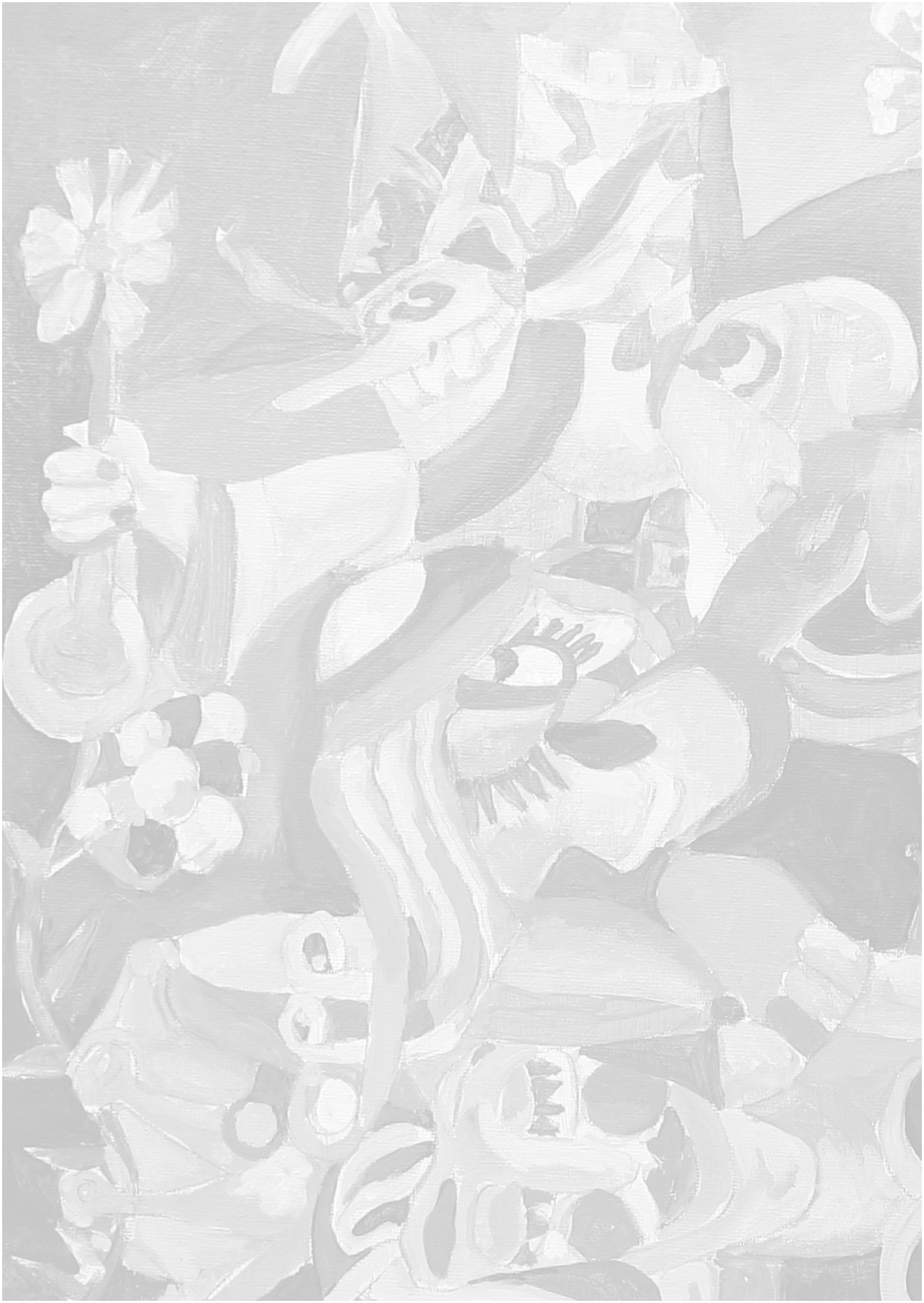
Table S2. DVT risk for *ANXA5* SNPs in the ACT study.

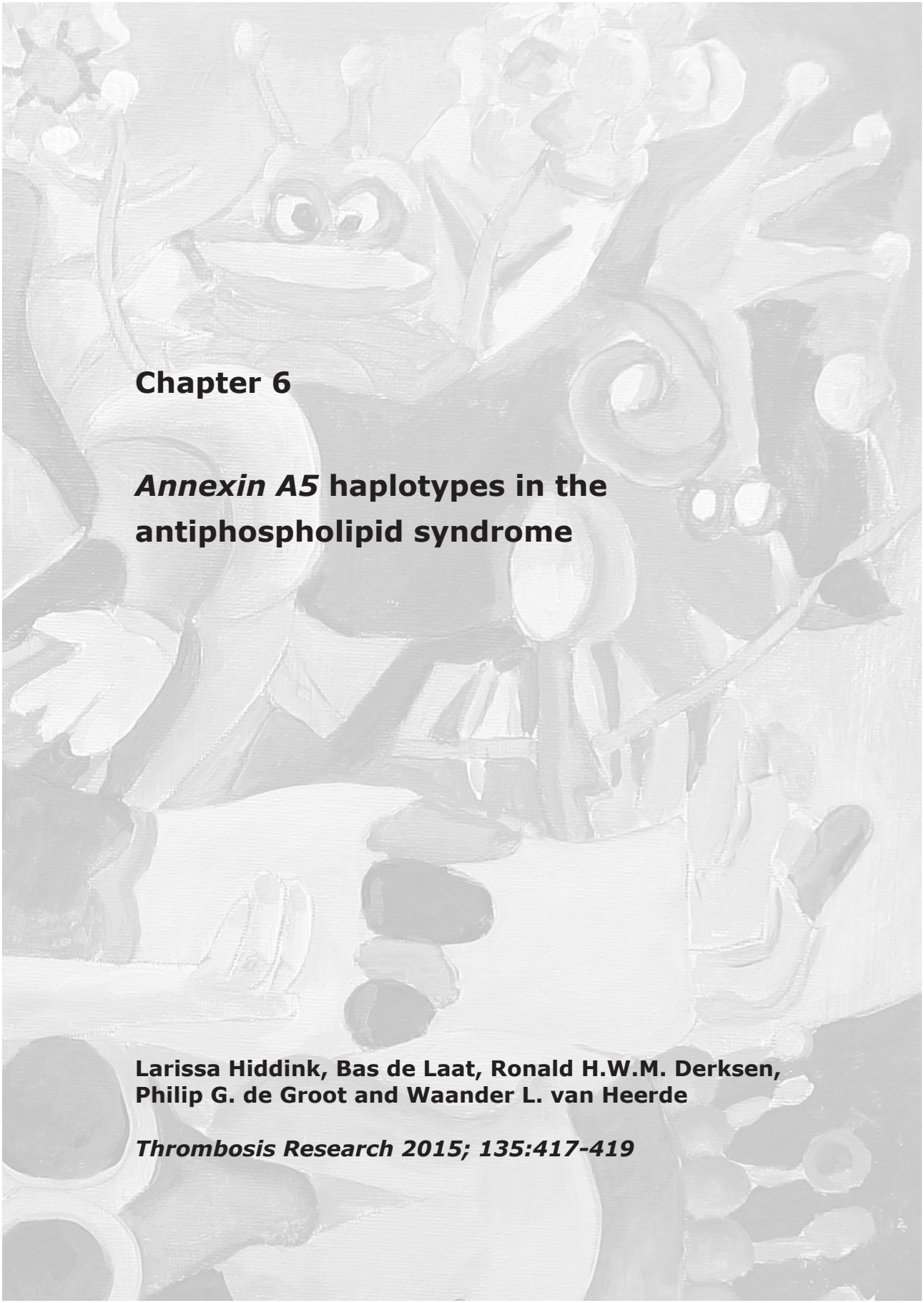
SNP	Geno-type	Patients (*n* = 148) *n* (%)	Controls (*n* = 267) *n* (%)	OR (95% CI)
SNP1 rs62319820	CC	125 (84.5)	221 (83.8)	1
	CT	22 (14.9)	45 (16.9)	0.9 (0.5-1.5)
	TT	1 (0.7)	1 (0.4)	1.8 (0.1-28.5)
	CT + TT	23 (15.5)	46 (17.2)	0.9 (0.5-1.5)
SNP2 rs112782763	GG	121 (81.8)	208 (77.9)	1
	GA	26 (17.6)	55 (20.6)	0.8 (0.5-1.4)
	AA	1 (0.7)	4 (1.5)	0.4 (0.05-3.9)
	GA + AA	27 (18.2)	59 (22.1)	0.8 (0.5-1.3)
SNP3 rs28717001	AA	99 (66.9)	171 (64.4)	1
	AC	46 (31.1)	82 (30.7)	1.0 (0.6-1.5)
	CC	3 (2.0)	14 (5.2)	0.4 (0.1-1.3)
	AC + CC	49 (33.1)	96 (36.0)	0.9 (0.6-1.3)
SNP4 rs28651243	TT	99 (66.9)	171 (64.4)	1
	TC	46 (31.1)	82 (30.7)	1.0 (0.6-1.5)
	CC	3 (2.0)	14 (5.2)	0.4 (0.1-1.3)
	TC + CC	49 (33.1)	96 (36.0)	0.9 (0.6-1.3)
SNP5 rs113588187	GG	121 (81.8)	208 (77.9)	1
	GA	26 (17.6)	55 (20.6)	0.8 (0.5-1.4)
	AA	1 (0.7)	4 (1.5)	0.4 (0.05-3.9)
	GA + AA	27 (18.2)	59 (22.1)	0.8 (0.5-1.3)
SNP6 rs1050606	TT	32 (21.6)	56 (21.0)	1
	TG	77 (52.0)	119 (44.6)	1.1 (0.7-1.9)
	GG	39 (26.4)	92 (34.5)	0.7 (0.4-1.3)
	TG + GG	116 (78.4)	211 (79.0)	1.0 (0.6-1.6)

All odds ratios (OR) were calculated with major alleles-only as the reference category (OR=1). DVT, deep venous thrombosis

Table S3. Frequency distribution of *ANXA5* SNPs and expected frequencies according to Hardy-Weinberg Equilibrium in NBS controls (n=1705).

Polymorphism		Risk allele frequency	Observed genotype	Expected genotype	*p-value*
SNP1	**rs62319820**	0.08			0.40
	CC		1445	147.5	
	CT		252	247	
	TT		8	10.5	
SNP5	**rs113588187**	0.11			0.88
	GG		1353	1352	
	GA		331	332	
	AA		21	20	
SNP6	**rs1050606**	0.51			0.26
	TT		394	405.5	
	TG		875	852	
	GG		436	447.5	
SNP7	**rs1131239**	0.11			0.95
	CC		1350	1350	
	CT		334	335	
	TT		21	21	





Chapter 6

Annexin A5 haplotypes in the antiphospholipid syndrome

Larissa Hiddink, Bas de Laat, Ronald H.W.M. Derksen, Philip G. de Groot and Waander L. van Heerde

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Antiphospholipid syndrome (APS) is an autoimmune disorder characterized by the persistent presence of antiphospholipid antibodies (aPLs) in plasma of patients with a history of vascular thrombosis and/or pregnancy morbidity [1,2]. Many pathogenic mechanisms have been described in APS, and in several of these mechanisms Annexin A5 (ANXA5) is suggested to play an important role [3]. ANXA5 is a Ca^{2+}- and phospholipid-binding protein highly expressed by vascular endothelium and placental syncytiotrophoblasts [4,5]. The protein exhibits strong anticoagulant properties in vitro and antithrombotic activities in animal models due to shielding of anionic phospholipids that normally catalyze the clotting cascade [6,7]. It has been shown that aPLs could reduce the quantity of ANXA5 on cell surfaces [8]. As a result of the aPL-mediated reduction of *ANXA5* expression, an acceleration of coagulation on cultured trophoblasts and endothelial cells has been demonstrated, which may be a potential mechanism for thrombotic events and pregnancy loss in APS [8,9].

In addition to the "immunologic" model of APS, Bogdanova and colleagues recently introduced the "genetic" model to explain obstetric complications in APS [10]. This latter model suggests that genetic variations in *ANXA5* could affect ANXA5 expression in placentas and thus the protective potential of ANXA5 against placental thrombosis. It has been proposed that carriage of the *ANXA5* M2 haplotype might predispose to obstetric complications as well as to the development of aPLs [10]. The M2 haplotype, comprising minor alleles of four promoter polymorphisms (rs112782763, rs28717001, rs28651243, rs113588187), has been linked to decreased *ANXA5* mRNA levels in placental tissues [11,12]. Several studies have shown an association of this haplotype with recurrent pregnancy loss and deep venous thrombosis [11,13,14]. Previously, we reported the four common haplotypes (H1 to H4) within the *ANXA5* gene upstream region and showed haplotype specific differences in plasma ANXA5 levels [15]. The major difference of our haplotypes with haplotypes described by *Bogdanova et al.* is that their wild-type N haplotype represents two haplotypes H1 and H2 associated with different plasma ANXA5 levels [15]. Furthermore, our H3 haplotype is an extension of the M2 haplotype [15].

The contribution of genetic variations in *ANXA5* to thrombotic risk or pregnancy morbidity in APS patients is poorly understood. In a well-defined cohort of patients with systemic lupus erythematosus (SLE), lupus-like disease (LLD) and primary APS [2,16], we sequenced the *ANXA5* promoter region and genotyped rs1131239 located in the *ANXA5* Kozak sequence to reconstruct the four common haplotypes [15]. An association of *ANXA5* haplotypes with thrombosis or pregnancy morbidity was investigated.

The study population consisting of 176 Caucasian patients (159 women/ 17 men) was recruited within the lupus clinic of the University Medical Centre Utrecht, the Netherlands. The clinical and immunological characteristics of the patients were described in detail previously [2,16]. Sixty patients (56 women/ 4 men; mean age 37.9±10.6 years; ±SD) met the Sapporo criteria

of APS [17]: 6 had primary APS; in 43 APS was associated with SLE and in 11 - with LLD. Of the 116 patients without APS (103 women/ 13 men; mean age 34.2±9.7 years; ±SD), 111 had SLE and 5 had LLD. Patients with SLE fulfilled the criteria of the American College of Rheumatology for the classification of SLE [18]. Among 60 APS patients, 46 persons had a history of thrombosis (in 22 arterial and in 31 venous). Sixty five of 159 female patients had been pregnant and 24 women with APS met the Sapporo criteria for pregnancy morbidity [17]. Serological detection of anticardiolipin antibodies (aCL), antibodies against β2-glycoprotein I and anti-ANXA5 antibodies was performed by ELISA assays as described [2,16]. Lupus anticoagulant (LAC) was measured by a PPT-LA and a diluted RVVT assay [2,16]. Plasma ANXA5 levels were determined by a "sandwich-ELISA" assay. Genomic DNA was extracted from peripheral mononuclear cells [16]. A 496-bp fragment of the *ANXA5* promoter was amplified by polymerase chain reaction. Purified amplicons were sequenced (BigDye Terminator v3.1 Cycle Sequencing Kit and ABI 3730 PRISM DNA Analyzer, Applied Biosystems). Single nucleotide polymorphism (SNP) rs1131239 was detected by restriction fragment length polymorphism [16].

The control group included population controls from the Nijmegen Biomedical study (NBS) [19]. For this study, we used the subset of 1819 individuals (900 men/ 919 women; mean age 62.0±10.3 years; ±SD) who served as controls in a genome-wide association study (GWAS) [20]. Of 919 female controls, 759 women had been pregnant. Informed written consent was obtained from all NBS controls. NBS participants were genotyped using the Illumina HumanHap CNV370-Duo BeadChip [20]. To reconstruct *ANXA5* haplotypes, the four *ANXA5* SNPs (rs62319820, rs113588187, rs1050606 and rs1131239) were extracted from genome-wide imputed SNP data using the Genome of the Netherlands as reference [21].

Categorical variables were compared by $\chi 2$ test and continuous variables - by unpaired t-test. The Hardy-Weinberg equilibrium for each SNP was tested by $\chi 2$ test. The association of *ANXA5* haplotypes with thrombosis or pregnancy morbidity was examined by contingency tables. Odds ratios (ORs) with 95% confidence intervals (CI) were calculated. Age and sex were included in the multivariate regression model. Two-sided p-values <0.05 were considered statistically significant.

Table 1 shows the individual SNPs and the four common *ANXA5* haplotypes. In controls, the genotypic distributions of the four SNPs were in Hardy-Weinberg equilibrium. One control person carrying a rare haplotype was excluded from further analyses. The three *ANXA5* haplotypes (i.e., H1, H2 and H4) were distributed similarly between groups of patients and between patients and controls (**Table 1** and **Table S1**). The frequency of *ANXA5* H3 was a slightly higher in APS patients compared to NBS controls ($p=0.053$).

Table 1. Individual SNPs and common haplotypes of the *ANXA5* gene.

	SNP1	**SNP2**	**SNP3**	**SNP4**	**SNP5**	**SNP6**	**SNP7**			
	g.-628C>T	*g.-467G>A*	*g.-448A>C*	*g.-422T>C*	*g.-373G>A*	*g.-302T>G*	*g.-1C>T*			
dbSNP ID	*rs62319820*	*rs112782763*	*rs28717001*	*rs28651243*	*rs113588187*	*rs1050606*	*rs1131239*			
Haplotype								***Frequency***		
								APS (n=60)	***SLE+LLD without APS (n=116)***	***NBS controls* (n=1818)***
H1	C	G	A	T	G	T	C	0.44	0.48	0.49
H2	C	G	A	T	G	**G**	C	0.34	0.32	0.32
H3	C	**A**	**C**	**C**	**A**	**G**	**T**	0.16	0.11	0.11
H4	**T**	G	**C**	**C**	G	**G**	C	0.06	0.09	0.08

Nucleotide numbering from the ATG initiation codon; SNP, single nucleotide polymorphism;
dbSNP indicates NCBI database for SNPs (http://www.ncbi.nlm.nih.gov/snp); minor alleles in bold and underlined.
APS, antiphospholipid syndrome; LLD, lupus-like disease; SLE, systemic lupus erythematosus; NBS, Nijmegen Biomedical Study.
In patients, *ANXA5* haplotypes were based on seven SNPs (SNP1-SNP7) as described [15].
In NBS controls, *ANXA5* haplotypes were constructed by using haplotype-tagging SNPs (SNP1, SNP5, SNP7) and SNP6, of which the T-allele is specific for haplotype H1.
*The rare *ANXA5* haplotype present in one control subject is not shown.

Table 2. The distribution of *ANXA5* haplotypes in thrombotic APS, obstetric APS and NBS controls.

Haplotype	Thrombotic APS, n=46, n (%)	NBS controls, n=1818, n (%)	Crude OR 95% (CI)	Obstetric APS, n=24, n (%)	Female NBS controls, n=759, n (%)	Crude OR 95% (CI)
Haplotype H1						
H1H1	10 (21.7)	422 (23.2)	0.8 (0.4-1.8)	4 (16.7)	183 (24.1)	0.6 (0.2-2.0)
H1Hx	22 (47.8)	929 (51.1)	0.8 (0.4-1.6)	13 (54.2)	390 (51.4)	0.9 (0.3-2.3)
H1H1/H1Hx	32 (69.6)	1351 (74.3)	0.8 (0.4-1.5)	17 (70.9)	573 (75.5)	0.8 (0.3-1.9)
Non-H1	14 (30.4)	467 (25.7)	1	7 (29.1)	186 (24.5)	1
Haplotype H2						
H2H2	6 (13.0)	191 (10.5)	1.3 (0.5-3.3)	1 (4.2)	76 (10.0)	0.5 (0.1-4.2)
H2Hx	20 (43.5)	796 (43.8)	1.0 (0.6-2.0)	14 (58.3)	323 (42.6)	1.7 (0.7-4.1)
H2H2/H2Hx	26 (56.5)	987 (54.3)	1.1 (0.6-2.0)	15 (62.5)	399 (52.6)	1.5 (0.6-3.5)
Non-H2	20 (43.5)	831 (45.7)	1	9 (37.5)	360 (47.4)	1
Haplotype H3						
H3H3	NP	23 (1.3)	NA	NP	9 (1.2)	NA
H3Hx	12 (26.1)	356 (19.6)	1.4 (0.7-2.8)	10 (41.7)	152 (20.0)	2.8 (1.2-6.4)
H3H3/H3Hx	12 (26.1)	379 (20.8)	1.3 (0.7-2.6)	10 (41.7)	161 (21.2)	2.6 (1.2-6.1)
Non-H3	34 (73.9)	1439 (79.2)	1	14 (58.3)	598 (78.8)	1
Haplotype H4						
H4H4	NP	9 (0.5)	NA	NP	6 (0.8)	NA
H4Hx	6 (13.0)	264 (14.5)	0.9 (0.4-2.1)	1 (4.2)	105 (13.8)	0.3 (0.04-2.0)
H4H4/H4Hx	6 (13.0)	273 (15.0)	0.8 (0.3-2.0)	1 (4.2)	111 (14.6)	0.2 (0.03-1.9)
Non-H4	40 (87.0)	1545 (85.0)	1	23 (95.8)	648 (85.4)	1

The results are expressed in odds ratios (OR). Hx indicates all haplotypes except for the one given; HxHx is the reference category (OR=1); NA, not applicable; NP, not present.

In patients with APS, none of the four *ANXA5* haplotypes was associated with thrombosis (either arterial or venous) both in an unadjusted model (**Table 2**) and after correction for age and sex (data not shown). Nor were *ANXA5* haplotypes associated with arterial and venous thrombosis separately. By selecting NBS controls without a history of thrombosis (i.e., myocardial infarction, deep venous thrombosis, pulmonary embolism, stroke) (n=1512), no significant differences were found either in the distribution of haplotypes between APS patients and controls (data not shown).

We further investigated an association of *ANXA5* haplotypes with obstetric APS (**Table 2**). Haplotype H1 and H2 were not associated with pregnancy morbidity, whereas carriers of *ANXA5* H3 (i.e., H3H3+H3Hx genotypes) had an increased risk compared to NBS controls (41.7% versus 21.2%; OR 2.6, 95% CI: 1.2-6.1, p=0.02). After adjustment for age, the risk for *ANXA5* H3 carriers remained increased (OR 2.8, 95% CI: 1.1-7.1, p=0.03). Haplotype H4 was non-significantly underrepresented in patients with obstetric APS compared to controls (4.2% versus 14.6%; p=0.2) (Table 2). Additional analyses performed after selection of female controls without a history of pregnancy loss (n=579) showed the same results. Only *ANXA5* H3 haplotype was associated with pregnancy morbidity, both in the unadjusted model (OR 2.8, 95% CI: 1.2-6.4, p=0.01) and after correction for age (OR 2.7, 95% CI: 1.1-6.7, p=0.03).

None of the *ANXA5* haplotypes was associated with the risk of developing anti-β2-glycoprotein I antibodies (IgG or IgM class) (**Table S2**). Moreover, no differences were found in the distribution of *ANXA5* haplotypes between aPL-positive (LAC, aCL) and aPL-negative patients (data not shown). In obstetric APS patients, *ANXA5* H3 was not associated with the risk of aPL development either. The prevalence of triple aPL positivity (LAC, aCL and anti-β2-glycoprotein I) was even slightly higher in non-H3 patients (6 from 14 women; 42.9%) compared to heterozygous H3 carriers (3 from 10 women; 30.0%) (p=0.5). In the total population, *ANXA5* haplotypes were not associated with plasma ANXA5 levels (**Table S3**).

An important result of this study was a possible association of *ANXA5* H3 with obstetric manifestations in APS patients. According to the National Center for Biotechnology Information database for SNPs, the percentage of subjects carrying the rs112782763A-allele and rs113588187A-allele, which are both specific for H3 haplotype, is around 20% in the European population. We found an approximately 2-fold higher prevalence of H3 carriers in obstetric APS patients compared to that in the European population and in Dutch population controls (NBS study). Although overrepresentation of *ANXA5* H3 in obstetric APS is in agreement with preliminary data published by *Bogdanova et al.* [10], we had no evidence to confirm the suggestion made by *Bogdanova et al.* [10] that carriage of the H3 haplotype could be related to an increased production of aPLs.

The main limitation of this study was the small sample size of the study population. Larger case-control studies should be used to validate our results regarding the possible impact of *ANXA5* haplotypes on thrombotic risk and pregnancy morbidity in APS patients. Additionally, a reason for cautious interpretation of our results could be the inflammatory condition induced by SLE. Therefore, a study should be confined to patients with primary APS.

In summary, our results indicate that *ANXA5* haplotypes do not contribute to thrombotic risk in APS patients. *ANXA5* H3 may be associated with an increased risk of pregnancy morbidity but not with an increased production of aPLs. Further investigations are warranted to assess the link between *ANXA5* H3 and obstetric APS as a better understanding of mechanisms involved in pregnancy-related complications may open new pathways for a personalized medicine approach.

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Conflict of interest
None declared.

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SUPPLEMENTAL DATA

Table S1. The distribution of *ANXA5* haplotypes in patients and controls.

Haplotype	APS, n=60 n (%)	SLE+LLD without APS, n=116, n (%)	NBS controls, n=1818, n (%)	*p*-value[1]	*p*-value[2]
Haplotype H1				0.4	0.6
H1H1	12 (20.0)	23 (19.8)	422 (23.2)		
H1Hx	29 (48.3)	66 (56.9)	929 (51.1)		
Non-H1	19 (31.7)	27 (23.3)	467 (25.7)		
Haplotype H2				0.8	0.8
H2H2	6 (10.0)	12 (10.3)	191 (10.5)		
H2Hx	29 (48.3)	50 (43.1)	796 (43.8)		
Non-H2	25 (41.7)	54 (46.6)	831 (45.7)		
Haplotype H3				0.2	0.053
H3H3	0	1 (0.9)	23 (1.3)		
H3Hx	19 (31.7)	24 (20.7)	356 (19.6)		
Non-H3	41 (68.3)	91 (78.4)	1439 (79.2)		
Haplotype H4				0.3	0.7
H4H4	0	0	9 (0.5)		
H4Hx	7 (11.7)	20 (17.2)	264 (14.5)		
Non-H4	53 (88.3)	96 (82.8)	1545 (85.0)		

APS, antiphospholipid syndrome; LLD, lupus-like disease; SLE, systemic lupus erythematosus; NBS, Nijmegen Biomedical Study.
1 Differences and significances in the distribution of *ANXA5* haplotypes between APS patients and SLE+LLD patients without APS.
2 Differences and significances in the distribution of *ANXA5* haplotypes between APS patients and NBS controls.
P-values are based on $\chi 2$ statistics.

Table S2. Association of *ANXA5* haplotypes with the presence of anti-β2-ycoprotein I antibodies

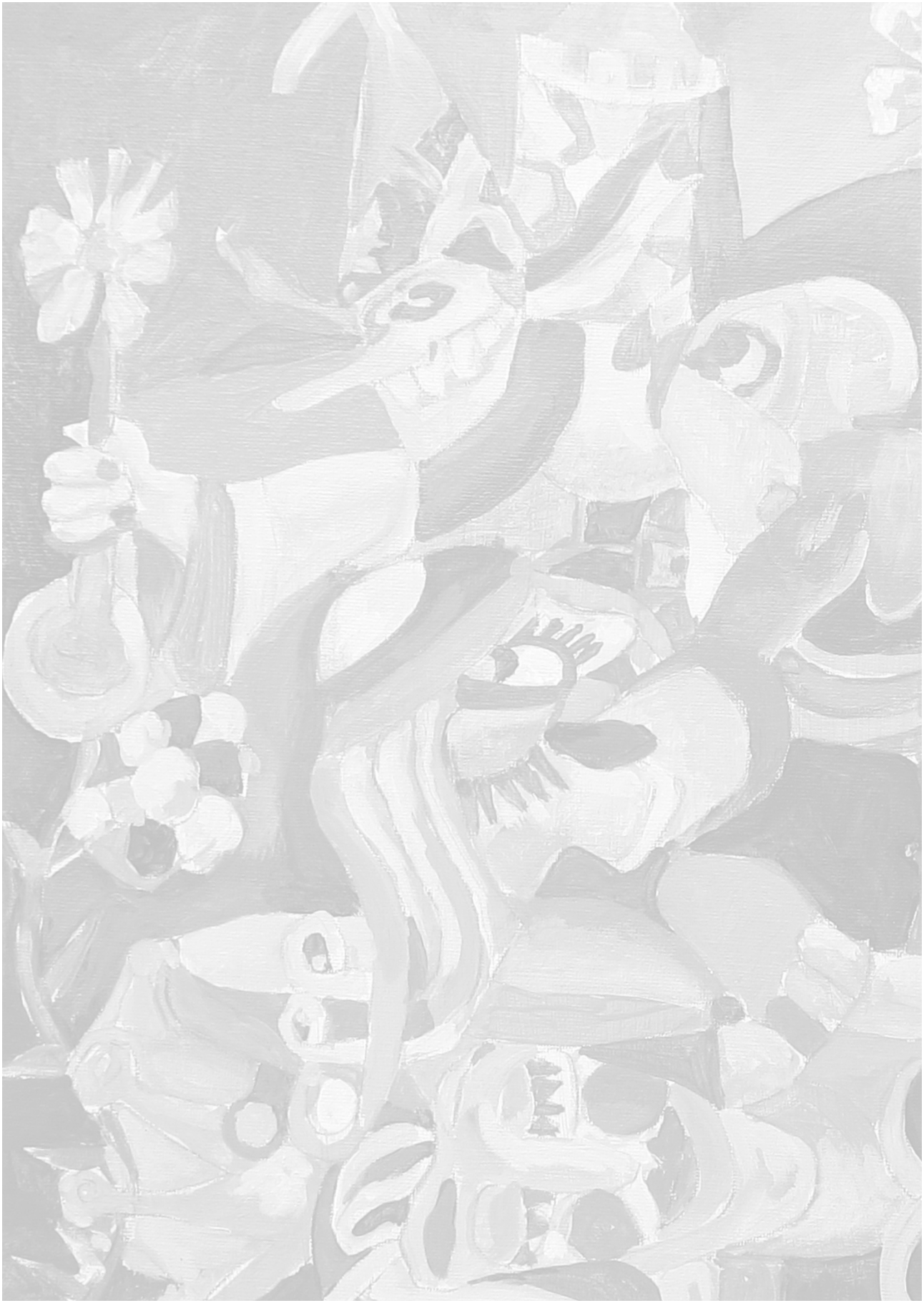
Haplotype	APS patients , n=60		p-value*	SLE+LLD patients without APS, n=116		p-value*
	Anti-β_2-GPI positive n=38, n (%)	Anti-β_2-GPI negative n=22, n (%)		Anti-β_2-GPI positive n=19, n (%)	Anti-β_2-GPI negative n=97, n (%)	
Haplotype H1			0.7			0.6
H1H1	7 (18.4)	5 (22.7)		5 (26.3)	18 (18.6)	
H1Hx	20 (52.6)	9 (40.9)		11 (57.9)	55 (56.7)	
Non-H1	11 (28.9)	8 (36.4)		3 (15.8)	24 (24.7)	
Haplotype H2			0.7			0.7
H2H2	3 (7.9)	3 (13.6)		1 (5.3)	11 (11.3)	
H2Hx	18 (47.4)	11 (50.0)		9 (47.4)	41 (42.3)	
Non-H2	17 (44.7)	8 (36.4)		9 (47.4)	45 (46.4)	
Haplotype H3			0.6			0.8
H3H3	NP	NP		NP	1 (1.0)	
H3Hx	13 (34.2)	6 (27.3)		3 (15.8)	21 (21.6)	
Non-H3	25 (65.8)	16 (72.7)		16 (84.2)	75 (77.3)	
Haplotype H4			0.6			0.8
H4H4	NP	NP		NP	NP	
H4Hx	5 (13.2)	2 (9.1)		3 (15.8)	17 (17.5)	
Non-H4	33 (86.8)	20 (90.9)		16 (84.2)	80 (82.5)	

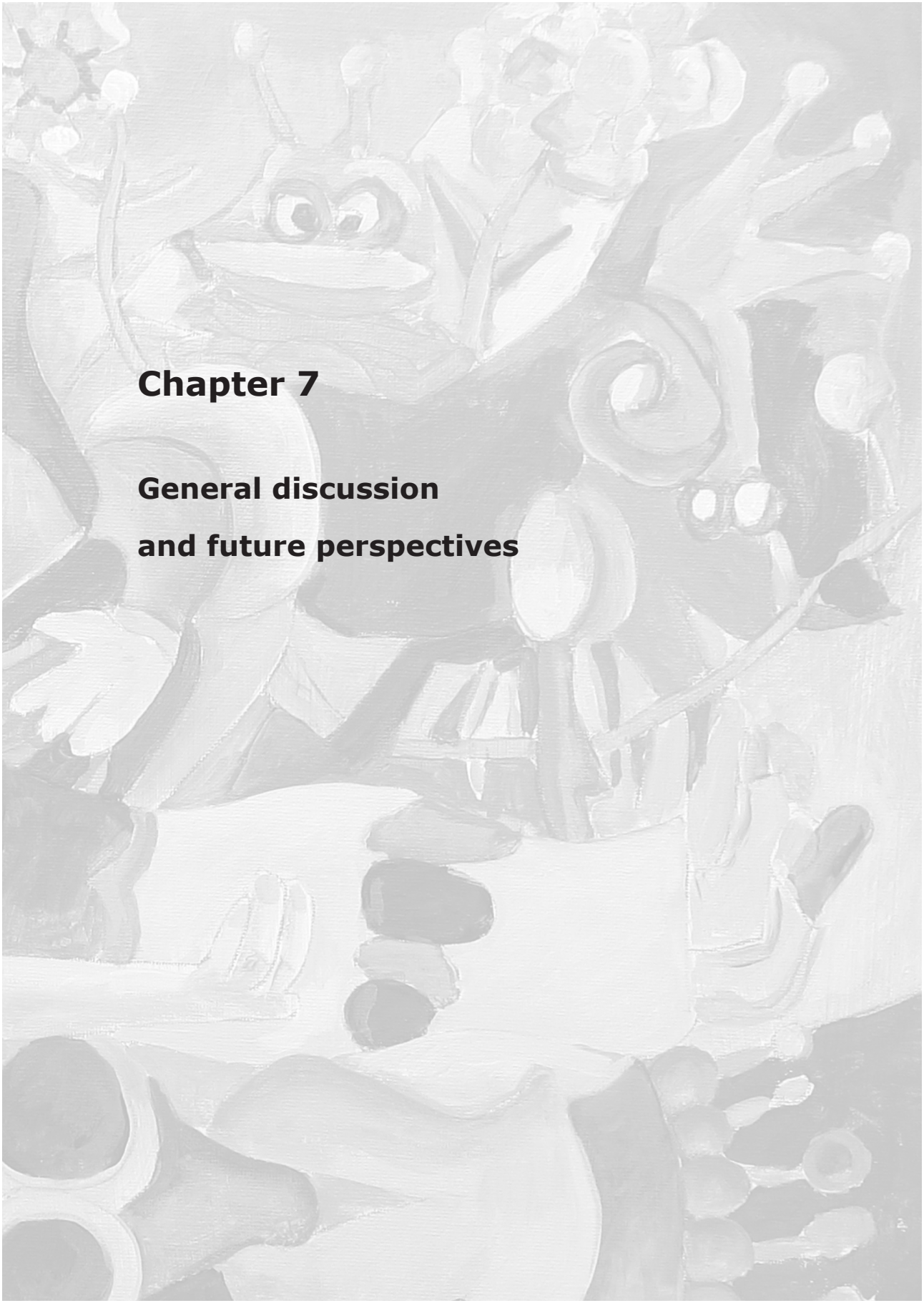
APS, antiphospholipid syndrome; LLD, lupus-like disease; SLE, systemic lupus erythematosus.
Anti-β_2-GPI, anti-β2-glycoprotein I antibodies of either IgM or IgG class; NP, not present.
*Based on $\chi 2$ statistics.

Table S3. Association of *ANXA5* haplotypes with plasma ANXA5 levels.

Haplotype	Number (%) n=176	Geometric mean ANXA5 (95% CI), µg/L
Haplotype H1		
H1H1	35 (19.9)	6.30 (4.71-8.33)
H1Hx	95 (54.0)	7.03 (6.17-8.08)
Non-H1	46 (26.1)	7.69 (6.30-9.49)
Haplotype H2		
H2H2	18 (10.2)	8.76 (6.11-12.68)
H2Hx	79 (44.9)	6.75 (5.75-8.00)
Non-H2	79 (44.9)	7.03 (5.99-8.17)
Haplotype H3		
H3H3	1 (0.6)	8.17
H3Hx	43 (24.4)	7.17 (6.05-8.50)
Non-H3	132 (75.0)	7.03 (6.17-8.00)
Haplotype H4		
H4Hx	27 (15.3)	7.75 (5.93-10.07)
Non-H4	149 (84.7)	6.94 (6.17-7.77)

Results are based on ln-transformed plasma ANXA5 levels. All values reported were reconverted to geometric means with the appropriate 95% confidence intervals.
Hx indicates all haplotypes except for the one given; CI, confidence interval.
No differences were found.





Chapter 7

General discussion and future perspectives

GENERAL DISCUSSION

Thesis objective

Annexin A5 (ANXA5), a calcium-dependent phospholipid-binding protein, exerts strong anticoagulant and antithrombotic properties as well as anti-inflammatory features. Due to its high affinity binding to phosphatidylserine (PS), ANXA5 is widely used as an imaging probe to visualize apoptosis non-invasively in animal models and in patients [1-3]. Besides its excellent imaging capacities, ANXA5 is also a therapeutic agent. The native ANXA5 as well as fusion proteins based on the structure of the native ANXA5 molecule have powerful antithrombotic capacities in animal thrombosis models [4-8]. More importantly, the inhibitory effects of ANXA5 are not associated with a bleeding tendency as compared to other anticoagulant therapies. However, full understanding of ANXA5-mediated anticoagulant effects is needed before this therapy can be applied to humans. One of the objectives of this thesis was to extend our knowledge about the mode of action of ANXA5-based anticoagulation on negatively charged phospholipids-exposing cell surfaces (chapter 2).

The history of ANXA5 started about 36 years ago from its isolation from placental tissues. Through the years, we learned a lot about exogenous ANXA5. The physiological significance of endogenous ANXA5, however, is still not completely understood. So far, only two annexins were related to a clinical phenotype, later termed as "annexinopathy." By the identification of abnormalities in ANXA5 expression in the antiphospholipid syndrome [9] and by the description of the M2 promoter haplotype within the *ANXA5* gene, associated with lower ANXA5 mRNA levels in placentas and placental thrombotic complications in some studies [10-13], we have made a little step towards understanding of biological role of endogenous ANXA5 in humans. With respect to the second objective of this thesis we investigated whether *ANXA5* genetic variations contribute to pathogenesis of thrombosis- (deep venous thrombosis, antiphospholipid syndrome) or inflammation-related diseases (atherosclerosis in familial hypercholesterolemia patients) in order to get more insight into the biological significance of endogenous ANXA5 in humans as well as to identify possible new biomarkers and their clinical implications (chapters 3, 4, 5 and 6).

ANXA5 lattices in ANXA5-mediated anticoagulation

In the 1980s, *Chris Reutelingsperger and colleagues* isolated ANXA5, earlier called a VAC (vascular anticoagulant protein), from human umbilical cord arteries and from the intima of bovine aorta, and demonstrated strong anticoagulant capacities of ANXA5 in vitro [14;15]. Thereafter, it has been shown that ANXA5 is a potent inhibitor of thrombus formation in vivo in a venous thrombosis model as well as in an arterial thrombosis model [4;5;16].

The anticoagulant properties of ANXA5 have been extensively studied on the surface of artificial and cellular membranes [15;17;18]. Its high affinity for negatively charged phospholipids, especially PS (dissociation constant [Kd] <0.2 nM), forms the basis for ANXA5 inhibitory activities [19;20]. ANXA5 binds with higher affinity to PS as compared to most coagulation

factors [20]. In this respect, ANXA5 interferes in blood coagulation through displacement of coagulation factors from procoagulant surfaces and thus inhibiting their complex formation.

Andree and colleagues introduced the idea that the ANXA5 anticoagulant mechanism is more complex than just displacement of coagulation factors from PS-exposing surfaces. They showed the incomplete displacement of FXa, FVa and prothrombin from the phospholipid surface by ANXA5 and proposed that the rigid ANXA5 clusters may reduce the lateral mobility of membrane-bound coagulation factors preventing thereby their complex formation [21]. Now it is known that membrane-bound ANXA5 molecules do not exist as monomers but assemble into trimers and ordered arrays of trimers [22-25]. However, the exact role of the 2D ordered arrays of ANXA5 in PS-depending coagulation reactions is still a subject of investigation. In **chapter 2**, we provided new insights into the role of ANXA5 lattices in ANXA5-mediated anticoagulant effects. We studied the interactions between labeled FVIII and FIX on PS-exposing cell surfaces applying a flow cytometric and microscopic FRET experimental approach, which is a well-known technique to study protein-protein interactions within a distance of 2-10 nm [26;27]. Our results indicate that pre-bound ANXA5 arrays (i.e., 2D ordered lattices) reduce but do not fully block the binding of coagulation factors to activated cells. That means that FIX and FVIII are able to bind to available PS molecules on the cell surface and that available anionic phospholipids could be located within the protein-free spaces of ANXA5 lattices. It is well established that on the surface of artificial membranes, ANXA5 assembles into open lattices containing large protein-free spaces (~9 nm in diameter) [22;28;29]. We believe that ANXA5 molecules may also form open arrays on cell surfaces and that coagulation factors or other proteins could be integrated within ANXA5 2D lattices. The second interesting aspect of our results concerns the reduced lateral mobility of PS-bound coagulation factors by ANXA5 arrays. We clearly showed that ANXA5, assembled into 2D ordered lattices, inhibits FIX-FVIII interactions (i.e., the tenase complex formation) already at low concentrations (5 nM), whereas ANXA1, which does not form 2D lattices, only reduced FIX-FVIII interactions under the same conditions. It should be noted that the binding of trimer-forming annexins (i.e., ANXA5, ANXA4 and ANXB12) to a phospholipid surface causes the rigidification of a cellular membrane and consequently creates the regions of diminished fluidity within the membrane [30;31]. In this regard, membrane-bound coagulation factors FIX and FVIII seemed to be restricted by rigid sheets of pre-bound ANXA5 to interact with each other and hence to form the tenase complex. However, our suggestion about reduced lateral diffusion of coagulation factors in the presence of ANXA5 lattices was not confirmed by other methods. FRAP (Fluorescence Recovery After Photobleaching) experiments, which were applied to study the mobility of FIX and FVIII on membrane surfaces, appeared to be not suitable most likely due to a high diffusion rate of coagulation factors on PS-exposing membranes (unpublished results). Fluorescence correlation spectroscopy (FCS) experiments would be very interesting in order to determine the mobility-related parameters of coagulation factors and to prove our suggestions.

This study was one of the first attempts towards understanding the role of ANXA5 organization on cell surfaces in ANXA5-mediated anticoagulant effects. Our work demonstrated that ANXA5 lattices inhibit tenase complex formation not only by shielding of PS but also presumably by reducing the lateral mobility of PS-bound coagulation factors. In pathological situations such as antiphospholipid antibody syndrome (APS), antiphospholipid antibodies could disrupt the ANXA5 anticoagulant shield on vascular endothelium or placental syncytiotrophoblasts [32;33] that may increase the mobility of coagulation factors and enhance their interactions ultimately leading to thrombin formation and an increased thrombotic risk. It is known that antiphospholipid antibodies belong to a heterogeneous population of autoantibodies meaning that only part of these autoantibodies may cause thrombosis through interaction with endogenous ANXA5 and through disruption of ANXA5 lattices. As an example, a monoclonal antiphospholipid antibody CIC15, which is responsible for pregnancy loss in mice, do not disrupt the ANXA5 2D ordered organization (AFM experiments) and do not inhibit ANXA5 anticoagulant properties [28]. Moreover, it is still a subject of debate whether antiphospholipid antibodies may promote thrombosis through displacing of ANXA5 from procoagulant cell surfaces and thus through reducing of ANXA5 concentrations on damaged cells [32;34-37]. We applied our flow cytometric FRET approach to examine whether antiphospholipid antibodies interfere in the binding of ANXA5 to PS-exposing cells and/or in ANXA5 2D lattices. Our preliminary data clearly showed that the 4F3 monoclonal anti-$ß_2$GPI antibody (provided by Prof. de Groot, University Medical Centre, Utrecht), directed against domain I of $ß_2$GPI, disturbs ANXA5 lattice formation on the surface of ionomycin-activated platelets but do not affect the binding of ANXA5 to activated platelets (unpublished results). On the other hand, monoclonal WAC2a anti-ANXA5 antibodies, which are directed against the phospholipid-binding sites of ANXA5 [38], reduced the binding of ANXA5 to activated platelets in a concentration-dependent manner but did not disturb pre-bound ANXA5 lattices on PS-exposing cell surfaces (unpublished results). Whether our FRET approach may contribute to understanding of prothrombotic properties of antiphospholipid antibodies requires further investigation. However, we believe that the knowledge provided herein could motivate future research and could be of clinical importance.

Endogenous ANXA5 and disease

The idea that ANXA5, the most abundant member of the annexin family, could contribute to the pathophysiology of human diseases is not new. It comes from the wide distribution of ANXA5 in human tissues, especially its high expression in placental syncytiotrophoblasts and vascular endothelium. The contribution of ANXA5 to human diseases is also based on ANXA5 anticoagulant, antithrombotic, anti-inflammatory and anti-apoptotic capacities. The first evidence for an antithrombotic role of endogenous ANXA5 in human morbidity was demonstrated by *Jacob H. Rand*, who linked the antiphospholipid antibody-mediated reduction of ANXA5 expression on the surface of endothelial cells and placental trophoblasts (”immunologic” model) to vascular thrombosis and pregnancy loss in patients suffering from antiphospholipid syndrome [9;39]. Later, Bogdanova et al. presented genetic

evidence for the association of reduced ANXA5 expression through carriage of the *ANXA5* M2 haplotype with recurrent pregnancy loss (defined as 2 or more consecutive pregnancy losses <20 weeks of gestation) ("genetic" model) [10]. Here we provided data linking genetic variations in *ANXA5* with inflammation-related and thrombosis-related disease conditions.

***ANXA5* genetic variations in atherosclerosis**

Atherosclerosis is a chronic inflammatory disease of the vascular wall consisting of two different distinct pathologic processes - conventional atherogenesis (i.e., the development of atheromatous plaques) at early stages and atherothrombosis (trigged by a disruption of the atherosclerotic plaques) at advanced stages of the disease [40-42]. Atherothrombosis is formed on an underlying vulnerable atherosclerotic lesion, which is characterized by an increased degree of inflammation and a high prothrombotic potential [43]. A rupture of the plaque's fibrous cap can trigger thrombus formation and subsequently acute obstruction of the blood vessels, manifesting as myocardial infarction or stroke [41].

ANXA5 has been found abundantly within human atherosclerotic plaques especially at the sites with high prothrombotic potential [44;45]. It has been suggested that endogenous ANXA5 can prevent plaque rupture by shielding damaged and/or activated cells and can inhibit propagation of thrombus formation [46-48]. However, little is known about the true effects of endogenous ANXA5 within atherosclerotic lesions. In contrast, recombinant ANXA5 has favorable effects in prevention of atherosclerosis in both in vitro and in vivo models. In proatherogenic ApoE-/- mouse models, administration of ANXA5 reduces plaque inflammation of advanced atherosclerotic lesions and vascular remodeling as well as improves endothelial function [49-51]. Furthermore, recombinant ANXA5 has been shown to inhibit uptake of oxidized LDL by macrophages, an important step in foam cell formation and acceleration of atherosclerosis [52]. Besides these effects, ANXA5 also reduces pro-inflammatory effects of oxidized LDL, such as induction of matrix metalloproteinase-9 or synthesis of leukotriene B4, which are both involved in plaque instability and plaque rupture [52;53]. Based on these findings, it has been suggested that recombinant ANXA5 could play a protective role in both atherogenesis and atherosclerotic plaque rupture [53].

In this regard, we hypothesized that *ANXA5* variations affecting ANXA5 expression levels within the arterial wall or in plasma could be associated with clinical features such as carotid IMT (intima-media thickness, a surrogate marker of atherosclerosis) and/or cardiovascular disease (CVD) risk. Reduced ANXA5 expression, for instance, may result in inefficient shielding of exposed procoagulant surfaces in the plaque and consequently in activation of coagulation. The hypercoagulability appears to have a protective effect against vascular injury within early atherosclerotic vessels on the one hand and to contribute to the formation of intraplaque thrombi and plaque instability in the advanced stages of the disease on the other hand [42;54]. Moreover, because of an extensive cross-talk between coagulation and inflammation [55], a prothrombotic environment may also

contribute to the maintenance of a persistent inflammatory state within the arterial wall, which in turn leads to more rapid progression of atherosclerosis and an increased risk of cardiovascular events. In this regard, two *ANXA5* common haplotypes, namely H3 and H4, could be of particular interest: *ANXA5* H3 (reported as M2 haplotype) is associated with reduced *ANXA5* mRNA expression in placental tissues and placental thrombosis in some studies [10-12;56] and *ANXA5* H4 is associated with higher plasma ANXA5 levels in controls (**chapter 3**). Whether *ANXA5* haplotypes really influence *ANXA5* expression within the arterial wall is still unknown.

In **chapter 4**, we presented data obtained from two independent patient cohorts with familial hypercholesterolemia (FH). The combination of the ASAP (Atorvastatin versus Simvastatin on Atherosclerosis Progression) trial and the GIRaFH (Genetic Identification of Risk factors in Familial Hypercholesterolemia) cohort allowed us to investigate the contribution of genetic variants in *ANXA5* and plasma ANXA5 levels to carotid IMT progression and CVD risk. However, our results provided no evidence to support our above mentioned hypothesis. We did not find any significant associations of *ANXA5* SNPs/haplotypes with carotid IMT and CVD risk. Plasma ANXA5 levels, which were partially determined by variations in *ANXA5*, did not correlate with carotid IMT parameters either. The result of this study is in contrast to the results of Ewing et al., who demonstrated a moderate association between two *ANXA5* intronic SNPs (rs4833229 and rs6830321) and the risk on restenosis in patients undergoing percutaneous coronary intervention for atherosclerosis [50]. The discrepancy with the study of Ewing et al. could be particularly explained by the pathophysiology of FH. Atherosclerosis is a complex and multifactorial disease. Although some studies succeeded to find genes and biomarkers related to atherosclerosis, there may be more studies that failed to find any association between genes and atherosclerosis or CVD risk. Furthermore, it is well established that FH patients are characterized by very high plasma LDL-C levels, which maintain a chronic inflammatory environment within the arterial wall and lead in turn to accelerated progression of intima-media thickness and a greatly increased risk for premature coronary artery disease [57]. Effects of elevated LDL-C levels in FH patients on the inflammatory reactions in the arterial wall should be more dominant than effects of endogenous ANXA5. Actually, we still do not know the real effects of endogenous ANXA5 in human atherosclerotic plaques. A protective role of endogenous ANXA5 in atherosclerosis development in double knock-out (ApoE-/- and *ANXA5* -/-) mouse models is currently under investigation. Moreover, it has been demonstrated that the antiphospholipid antibody-mediated reduced binding of ANXA5 to endothelial cells is associated with atherosclerotic changes and supposed to be a mechanism for atherothrombosis in patients with systemic lupus erythematosus (SLE) [44]. As SLE patients are known to have an accelerated, early atherosclerosis, it should be interesting to investigate the role of *ANXA5* genetic variations in this patient population.

Taken together, although exogenous ANXA5 functions as a potent antiatherogenic and antiatherothrombotic agent, the physiological significance of endogenous ANXA5 in atherosclerosis have to be proven.

***ANXA5* genetic variations in thrombosis-related pathologies**

Thrombosis, the formation of a clot within a blood vessel, may occur in arteries (i.e., **arterial thrombosis**), leading to myocardial infarction or stroke as well as in the deep veins (i.e., **deep venous thrombosis, DVT**), most often in the large veins of the legs and can be associated with pulmonary embolism (PE; i.e., a blood clot in the lungs) [58;59]. DVT and PE are collectively termed as venous thromboembolism (VTE). Thrombosis, which occurs in placenta (i.e., **placental thrombosis**), is responsible for some pregnancy complications such as pregnancy loss and preeclampsia.

ANXA5 is linked to thrombotic disease through the antiphospholipid syndrome (APS). In 1997, *Rand et al.* demonstrated that the antiphospholipid antibody-induced reduction of ANXA5 on the surface of endothelial cells and trophoblasts lead to acceleration of blood coagulation [39]. It has been suggested that ANXA5 may have an antithrombotic role in vivo and that underexpression of ANXA5 on cell surfaces may account for vascular thrombosis and pregnancy loss in APS ("immunologic" model). The ANXA5 resistance assay [60] presented later confirmed that patients with the APS-associated vascular thrombosis [60] and pregnancy morbidity [61] had reduced ANXA5 anticoagulant activity.

Hypothetically, genetic variations within the *ANXA5* gene could also affect expression of ANXA5 on cell surfaces contributing thereby to a local procoagulant state ("genetic" model). With the description of the *ANXA5* M2 promoter haplotype, which appears to regulate the transcription of *ANXA5*, several studies have been performed to investigate an association between the M2 haplotype and DVT risk. *Grandone and colleagues* demonstrated that carriers of the *ANXA5* M2 haplotype had a 3.4-fold increased risk of pregnancy-related venous thrombosis in a study of 83 cases and 195 controls (13). The same authors showed that the M2 haplotype is associated with thrombotic risk (OR 2.2, 95% CI: 1.3-3.7) in the general population from Southern Italy (231 cases and 382 controls) [62]. In **chapter 5**, we evaluated these findings and clearly demonstrated that *ANXA5* SNPs/haplotypes do not contribute to DVT risk in the Dutch general population (148 cases, 267 hospital controls and 1705 population controls). Our finding is in line with a recent Norwegian study in which no associations were found between genetic variations in *ANXA5* (i.e., the M1, M2 haplotypes and six tag-SNPs) and the risk of pregnancy-related venous thrombosis (313 cases and 353 controls) [63]. It should be noted that there is the discrepancy in calling of haplotypes. The wild type N haplotype of *Bogdanova et al.* [10], which is used as the reference category in many studies, actually includes two haplotypes: H1 (really wild type haplotype) and H2 (associated with lower plasma ANXA5 levels) (**chapter 3**). Based on this aspect, *Bogdanova's* N haplotype (the combination H1+H2) should not be used as one reference category.

A possible explanation for the discrepancy with previously published results of the Italian study of *Grandone et al.* could be differences in genotype/haplotype frequencies in the controls. It is well established that genotype/haplotype frequencies vary between ethnic or geographic groups. Based on more than 270.000 SNPs, *Nelis et al.* described the European genetic structure identifying four areas, namely: 1) Central and Western Europe, 2)

the Baltic countries, Poland and Western Russia, 3) Finland, and 4) Italy [64]. According to our results, a percentage of Dutch controls carrying the ANXA5 H3 haplotype (22.1% in ACT controls, 20.7% in NBS controls (**chapter 5**) and 21.4% in healthy controls (**chapter 3**)) is considerably higher compared to that in Italian controls (reported as M2 haplotype; 14.4% and 16.6%) [13;62] and in Norwegian controls (reported as M2 haplotype; 15.1%) [63]. However, the haplotype frequency of *ANXA5* H3 found in our studies is similar to that observed in European population-based controls from the dataset of 1000 Genomes Project (22.4%; Europe is represented by Utah residents with Northern and Western European ancestry) and therefore our result is in line with the findings of Nelis et al. With regard to the differences in haplotype frequencies between Dutch and Italian controls, they are also in accordance with the European genetic structure. Hence, the *ANXA5* M2 haplotype may represent a new thrombophilic risk factor for pregnancy-related venous thrombosis in Italian patients if further studies confirm this result.

Another explanation for our inability to find any significant associations with DVT in this study could be the current study design. The question is whether DNA sequencing of the coding regions and exon-intron boundaries of *ANXA5* besides the sequencing of the *ANXA5* promoter used in this study could be a right approach to find other genetic variations in *ANXA5* predisposing to DVT. In this regard, in 2012 Lotta et al. implemented the next-generation DNA sequencing strategy for sequencing of the coding regions of 186 hemostatic/proinflammatory genes, including the *ANXA5* gene, in Italian patients with idiopathic DVT [65]. Although *ANXA5* SNP rs2306416 (57-bp downstream from the ATG codon) belonged to the top-5 variants, this variation was not associated with DVT in the replication study. That means that even with a different study design, SNPs in the *ANXA5* gene are presumably not related to venous thrombosis.

In **chapter 6**, we evaluated patients with autoimmune disorders and we found no evidence for an association between *ANXA5* haplotypes and thrombosis risk in a subset patients with antiphospholipid syndrome (APS) either. The four *ANXA5* haplotypes were distributed similarly between APS patients (n=46) and a large group of population controls (n=1818). Taken together, in the Dutch population, genetic variations in *ANXA5* do not contribute to the risk of thrombosis in the general population and in patients with APS.
The hemostatic system also plays an important role in the pathogenesis of pregnancy complications, such as **APS-associated pregnancy morbidity.** ANXA5, which is abundantly present on the apical surfaces of placental syncytiotrophoblasts and shields anionic phospholipids externalized during trophoblast differentiation/fusion, has been suggested to play an antithrombotic role in the maintenance of blood fluidity in the placenta [66]. A reduction of the ANXA5 shield either antiphospholipid antibody-mediated or through carriage of the *ANXA5* M2 haplotype ("immunologic" model versus "genetic" model) was suggested to be responsible, at least in part, for the thrombophilic state at the syncytiotrophoblast surface and to explain some pregnancy complications [67-70]. It should be noted that the

association of the M2/*ANXA5* allele with reduced placental *ANXA5* mRNA levels was only demonstrated by Bogdanova et al. [11] but not observed by de Jong et al. [71]. Interestingly, the latter research group also found that the minor rs62319820T-allele, which is unique for the *ANXA5* H4 haplotype (an extension of the M1 haplotype), was associated with increased placental *ANXA5* mRNA expression [71]. In our previous report, we demonstrated that the rs62319820T-allele is a major contributor to higher plasma ANXA5 levels in healthy controls (**chapter 3**). For that reason, the rs62319820T-allele as a part of the M1 haplotype could not be associated with reduced *ANXA5* promoter activity as published by *Bogdanova et al.* [10] and it would be relevant to revisit this finding. The clinical relevance of *ANXA5* H4 (an extension of the M1 haplotype) is still unknown. In chapter 6, we investigated the contribution of the *ANXA5* haplotypes to pregnancy morbidity associated with APS. According to the revised Sapporo clinical criteria for APS, the term obstetric APS is defined as a previous unexplained recurrent first trimester loss and/or mid-trimester and third-trimester intrauterine death and/or severe preeclampsia, placental abruption, or intrauterine growth restriction [72]. Here we demonstrated overrepresentation of *ANXA5* H3 (an extension of the M2 haplotype) in obstetric APS women compared to female controls (24 cases and 759 controls; 41.7% versus 21.2%). This result is in line with the preliminary data of Bogdanova et al., showing enrichment of the M2 haplotype (36.7%) in a small cohort of patients with obstetric APS (n=30) [69]. The *ANXA5* H4 haplotypes associated with increased plasma ANXA5 levels was non-significantly underrepresented in obstetric APS compared to controls. Although our results suggest that *ANXA5* H3 is a risk factor for pregnancy morbidity associated with APS, replication in a larger patient cohort is needed to confirm these results. It would also be interesting to investigate whether *ANXA4* H4 plays a protective role in pregnancy complications.

FUTURE PERSPECTIVES

ANXA5 genetic variations as biomarker in human pathology

The term biomarker has been defined by the National Institutes of Health Biomarkers Definitions Working Group as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" [73].

Should genetic variations in *ANXA5*, a subject of our interest, serve as biomarkers in human pathology? Although the biological role of endogenous ANXA5 remains to be proven, we have evidence from in vitro and in vivo studies to suggest that endogenous ANXA5 plays an antithrombotic role in placental environment as well as in the vasculature [39;60;66;67]. Furthermore, in 2007 genetic evidence was given for the association between the *ANXA5* M2 promoter haplotype and recurrent pregnancy loss in German females [10]. It was suggested that the hypofunctional M2 allele may promote the procoagulant state in placental tissues and that the M2 haplotype could represent a new hereditary factor for obstetric complications and recurrent pregnancy loss [12;74]. An association of the *ANXA5* M2 haplotype with recurrent pregnancy loss was further demonstrated in Italian

[12], Japanese [56] and Bulgarian women [75], but not in subjects from East China [76] and also not in a second cohort of Japanese women [77].
It should be noticed that some issues require further attention. First, the percentage of M2 haplotype carriers among German controls is considerably lower (8.2% in Munster fertile controls; 14.4% in Munich fertile controls; 15.4% in PopGen population controls) [10;78] than the European population frequency based on the 1000 Genomes Project (22.4%; Europe is represented by Utah residents with Northern and Western European ancestry). According to Nelis et al. who described the European genetic structure [64], the frequency of the M2 haplotype in control subjects from Germany belonging to Central and Western Europe should be comparable with the European population frequency estimates. The second subject that needs attention in the German association studies is that there is a significant deviation from Hardy-Weinberg equilibrium in two control groups (Munich and Munster fertile controls), mainly due to an excess of M2 homozygotes [10;75;78]. Third, the association of the M2 haplotype with reduced placental *ANXA5* mRNA levels was only reported by *Bogdanova et al.* and was not confirmed in a recent study [71]. Furthermore, besides ANXA5, many other annexins are also present in placental tissues [79;80]. It should be kept in mind that changes in expression levels of one individual annexin could affect the expression of other members of the annexin family [81]. Imagine that the M2 haplotype (our H3 haplotype) really leads to downregulation of ANXA5 expression levels in placenta, and then it would be interesting to investigate if it could be compensated by the upregulation of other individual annexins and what is a collective protective role of annexins present in placenta in pregnancy complications. We therefore think that further work is required to understand above mentioned issues and revisit some results from in vitro experiments before we can conclude that the *ANXA5* M2 haplotype undoubtedly represents a factor predisposing to pregnancy complications and that it could be a biomarker for pregnancy failure. If more supportive evidence can be found for an association between the *ANXA5* M2 haplotype and pregnancy loss, screening for carriage of *ANXA5* M2 will help to identify a subgroup of women with an increased risk of pregnancy failure who might benefit from better management and personalized treatment. Consequently, based on disease mechanisms in women carrying *ANXA5* M2 rather than the disease outcome, recombinant ANXA5 and related compounds may be applied as novel therapeutic approaches for the treatment of pregnancy loss and other pregnancy complications. We hope that an increased understanding of ANXA5-mediated anticoagulant effects provided in this thesis will be of clinical significance contributing to further improvements in the therapeutic regimens.
Regarding the contribution of *ANXA5* variations to the risk of thrombosis in the general population or cardiovascular events in a patient cohort with accelerated atherosclerosis (familial hypercholesterolemia), our work did not reveal any associations between variants in the *ANXA5* gene upstream region and clinical parameters. That means that *ANXA5* variations could not serve as a thrombosis risk marker.

CONCLUSION

My PhD project was focused on two main aspects of ANXA5:
I) to extend our knowledge about ANXA5-mediated anticoagulant effects on cell surfaces exposing negatively charged phospholipids, especially a role of ANXA5 lattices in PS-depending coagulation reactions; II) to shift our focus from exogenous ANXA5 to understanding of physiological significance of endogenous ANXA5 in inflammation- and thrombosis-related pathologies.
We clearly showed that ANXA5 is assembled into ordered lattices on PS-exposing cell surfaces and that ANXA5 lattices inhibit tenase complex formation not only by shielding of PS but also likely by reducing the lateral mobility of membrane-bound coagulation factors.
We described four common *ANXA5* haplotypes in the *ANXA5* gene upstream region, of which two haplotypes influence plasma ANXA5 levels in controls. In atherosclerosis, which is a chronic inflammatory disease of the vascular wall, *ANXA5* variations and plasma ANXA5 levels are not associated with intima-media thickness parameters (a marker of atherosclerosis) or cardiovascular disease risk in two cohorts of familial hypercholesterolemia patients. The *ANXA5* polymorphisms did not contribute to deep venous thrombosis risk in the general population nor did they affect the risk of thrombosis (either arterial or venous) in a subset of patients with antiphospholipid syndrome. However, the *ANXA5* H3 haplotype was associated with obstetric manifestations in women suffering from antiphospholipid syndrome, a finding that merits further investigation.

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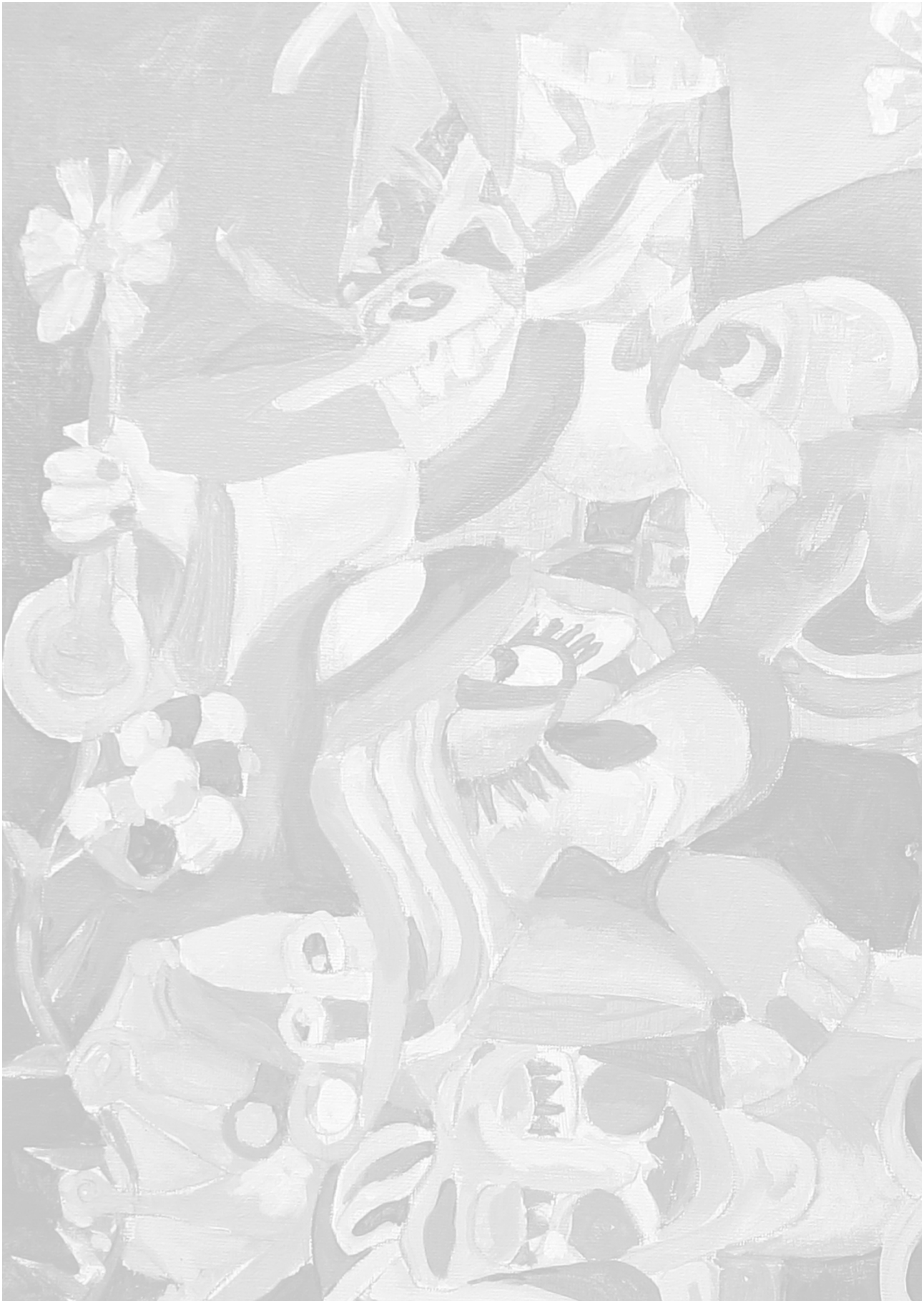

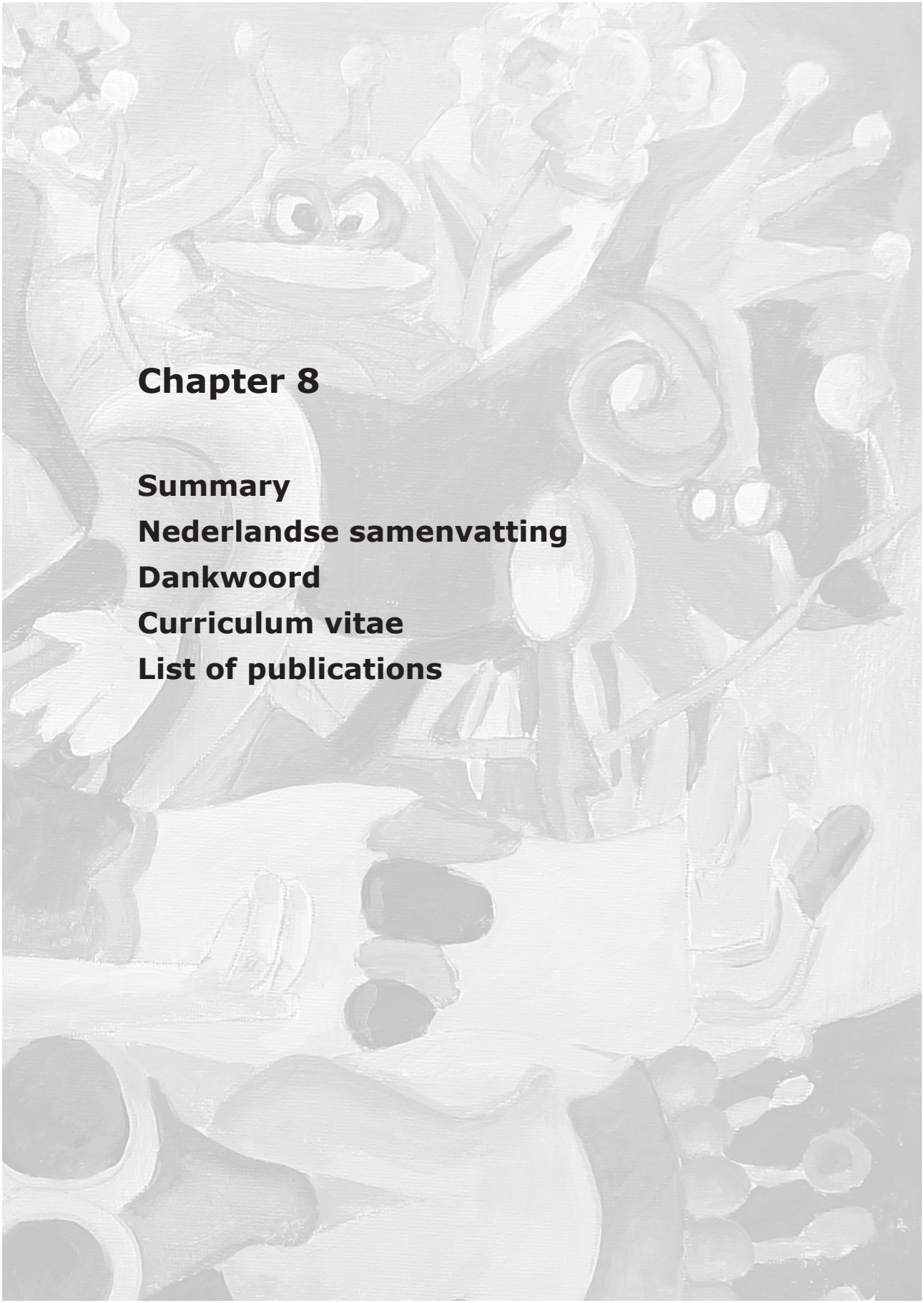

Chapter 8

Summary
Nederlandse samenvatting
Dankwoord
Curriculum vitae
List of publications

SUMMARY

This thesis was focused on two main aspects: I) to extend our knowledge about ANXA5-mediated anticoagulant effects on cell surfaces exposing negatively charged phospholipids; II) to understand the physiological significance of endogenous ANXA5 in inflammation- and thrombosis-related human pathologies.

ANXA5 lattices interfere in the tenase complex formation on PS-exposing cells

In **chapter 2**, we investigated the ANXA5 self-association on model membranes as well as on PS-exposing cell surfaces and tried to understand the role of ANXA5 lattices in PS-depending coagulation reactions. A flow cytometric and microscopic Fluorescence Resonance Energy Transfer (FRET) approach was used to visualize ANXA5 interactions. We clearly demonstrated that upon binding to ionomycin-activated platelets and ionomycin-stimulated COS1 cells, ANXA5 showed a high FRET signal, an indicator of membrane-bound structured arrays. We also showed that ANXA5 organization on the surface of artificial membranes is based on multiple ANXA5 interactions (a two-step FRET approach) and that these ANXA5-ANXA5 interactions do not grow in a three-dimensional direction independent of anionic phospholipids (experiments with amine beads). Thereafter, we used the same FRET experimental approach to visualize interactions between FIX and FVIII of the intrinsic pathway of blood coagulation. FIX-FVIII interactions were inhibited by low concentrations (5 nM) of pre-bound ANXA5. By contrast, labeled ANXA1, which binds to PS without forming 2D lattices, only partly affected FIX-FVIII interactions. From these observations, we proposed that besides the shielding of negatively charged phospholipids, the organization of ANXA5 on PS-exposing cell surfaces is also relevant in ANXA5-based anticoagulation. In the presence of ANXA5 arrays but not the ANXA1 clusters, PS-bound coagulation factors FIX and FVIII were restricted to interact with each other to form the tenase complex. Here, we provided evidence that pre-bound ANXA5 arrays interfere in the tenase complex formation (and possibly in the assembly formation of other coagulation factors) not only by shielding of PS molecules but presumably also by reducing the lateral mobility of PS-bound coagulation factors.

Genetic variations in the *ANXA5* gene and plasma ANXA5 levels

In **chapter 3**, we described common haplotypes (H) within the *ANXA5* gene upstream region and their associations with plasma ANXA5 levels in Dutch healthy individuals. In comparison with three *ANXA5* promoter haplotypes previously reported by *Bogdanova et al.* (9), we showed the presence of four common haplotypes H1-H4 (haplotype frequency >1%) within the *ANXA5* gene upstream region. We additionally demonstrated that our haplotypes H1 and H2, the two major haplotypes, included Bogdanova's wild-type haplotype N. Importantly, circulating ANXA5 levels were shown to be influenced by *ANXA5* genetic variants. Here we provided evidence for the association of haplotype H2 with decreased plasma ANXA5 levels and

haplotype H4 (tagged by SNP rs62319820) – with increased plasma ANXA5 levels.

Our finding regarding an association of *ANXA5* haplotype H2 and haplotype H4 with lower and higher plasma ANXA5 levels, respectively, was further confirmed in the patient cohort with atherosclerosis (**chapter 5**). Unfortunately, plasma ANXA5 levels were not of clinical importance as no correlation was found with any of the used phenotypic or clinical outcome parameters (i.e., carotid IMT, cholesterol levels and an inflammation marker, hs-CRP). Furthermore, in patients with autoimmune disorders (**chapter 6**), plasma ANXA5 levels were not influenced by *ANXA5* genetic variations nor were they associated with thrombosis or miscarriage.

Relevance of *ANXA5* genetic variations in (cardio)vascular disease In **chapter 4**, we investigated whether ANXA5, which possesses antiatherogenic properties, could serve as a biomarker in familial hypercholesterolemia (FH) patients with underlying atherosclerosis. The important characteristics of FH are elevated low-density lipoprotein cholesterol (LDL-C) levels and an increased risk of premature cardiovascular disease (CVD). We studied a possible relationship of *ANXA5* genetic variations and/or plasma ANXA5 levels with carotid IMT (intima-media thickness; a marker of atherosclerosis) in FH patients from the ASAP (Atorvastatin versus Simvastatin on Atherosclerosis Progression) trial. We additionally studied the contribution of *ANXA5* genetic variations to CVD risk in a large group of FH patients participating in the GIRaFH (Genetic Identification of Risk factors in Familial Hypercholesterolemia) study. We demonstrated that *ANXA5* SNPs and haplotypes H1-H4 did not contribute to the age-related IMT progression. Interestingly, *ANXA5* haplotype H3 tended to be associated with more rapid carotid IMT increase with age, but this association did not reach the Bonferroni-corrected statistical significance level. Furthermore, *ANXA5* genetic variations were not associated with CVD risk. The data obtained from two independent cohorts of FH patients indicate that both common genetic variants in *ANXA5* and plasma ANXA5 levels are not associated with carotid IMT parameters or CVD risk. That also means that ANXA5 could not serve as a biomarker in atherosclerosis, anyway, not in FH patients.

In **chapter 5**, we evaluated whether *ANXA5* promoter SNPs and/or haplotypes H1-H4 contribute to the risk of deep venous thrombosis (DVT) in the Dutch general population. We did not find any association between individual SNPs or common haplotypes H1-H4 and an increased risk for DVT. The genotype and haplotype groups were equally distributed among patients with newly diagnosed DVT and hospital controls without previous VTE (Amsterdam Case-control Thrombophilia (ACT) study) as well as among DVT patients and a large group of population controls (Nijmegen Biomedical Study). Our study was adequately powered to detect genotype- and/or haplotype-specific differences. Therefore, we concluded that *ANXA5* genetic variants do not contribute to DVT risk in the Dutch population.

There is plenty of evidence for the involvement of ANXA5 in the antiphospholipid syndrome (APS), an autoimmune disorder characterized by the persistent presence of antiphospholipid antibodies in plasma of patients with a history of vascular thrombosis and/or pregnancy morbidity. The contribution of genetic variations in *ANXA5* to thrombotic risk or pregnancy morbidity in APS patients is still poorly understood. In **chapter 6**, we demonstrated that *ANXA5* haplotypes H1-H4 did not contribute to thrombotic risk in APS patients. However, *ANXA5* H3 was overrepresented in women with obstetric APS, a subject that could be of clinical importance. None of the *ANXA5* haplotypes was associated with an increased production of antiphospholipid antibodies or with plasma *ANXA5* levels. Whether *ANXA5* H3 could represent a biomarker in pregnancy-related complications in APS, needs further investigation.

In **chapter 7**, we discussed our results presented in chapters 2 to 6.

SAMENVATTING

Dit proefschrift beschrijft de fysiologische betekenis van Annexine A5 (ANXA5). ANXA5 is één van de meest voorkomende eiwitten binnen de annexine familie die in totaal uit 12 humane annexines bestaat. ANXA5 komt veel voor in de placenta en in endotheelcellen. Evenals de andere eiwitten binnen de annexine familie bindt ANXA5 in aanwezigheid van Ca^{2+}-ionen aan een negatief geladen fosfolipidenoppervlak. Negatief geladen fosfolipidenoppervlakken bestaan uit negatief geladen fosfatidylserine (PS) moleculen en komen tot expressie bij cel activatie en celdood. We onderscheiden twee vormen van celdood; apoptose, een geprogrammeerde vorm van celdood, en necrose, een ongereguleerde vorm. ANXA5 heeft van alle humane annexines de hoogste affiniteit voor negatief geladen fosfolipiden. ANXA5 bindt met hoge affiniteit aan PS-exposerende celoppervlakken en schermt hierdoor de negatief geladen fosfolipiden af. Vanwege deze uitstekende bindingscapaciteiten is ANXA5 zeer geschikt om celdood in diermodellen en in patiënten aan te tonen en daarnaast zorgt dit mechanisme ook voor de antistollende en anti-inflammatoire activiteiten van dit eiwit. In trombose diermodellen zorgen het natieve ANXA5 en ANX5-bevattende fusie-eiwitten voor sterke antitrombotische effecten. Belangrijk is dat de ANXA5 anticoagulante effecten niet geassocieerd zijn met een verhoogde kans op bloedingscomplicaties. Dit is in tegenstelling tot andere antistollingsmiddelen die wel een verhoogde bloedingsneiging veroorzaken. Voordat ANXA5 klinisch toepasbaar zal zijn is het echter van groot belang de klinische betekenis van ANXA5 beter te begrijpen waaronder het exacte anticoagulante werkingsmechanisme van ANXA5.

Ongeveer 36 jaar geleden werd ANXA5 voor het eerst geïsoleerd uit placentaire weefsels. Door de jaren heen hebben we veel geleerd over exogeen ANXA5. De fysiologische betekenis van endogeen ANXA5 is echter nog niet volledig begrepen. Ook met betrekking tot de pathologie van annexines is nog veel werk te verzetten. De door *Rand et al.* geïntroduceerde term "annexinopathy" beschrijft slechts twee annexine (ANXA2 en ANXA5)-gerelateerde ziektebeelden waarbij de afwijkingen in de expressie levels centraal staan. Zo is een toegenomen expressie van ANXA2 in leukocyten gelinkt aan bloedingscomplicaties bij acute promyelocyten leukemie. Bij patiënten lijdend aan het antifosfolipidensyndroom is de verminderde ANXA5 expressie in de placenta en endotheelcellen geassocieerd met vasculaire trombose en herhaalde miskramen. Met de recente beschrijving van het M2 haplotype binnen het *ANXA5* gen, dat geassocieerd is met de lagere ANXA5 mRNA levels in placenta en placentaire trombose, hebben we een kleine stap gezet in het begrijpen van de (patho)fysiologische rol van endogeen ANXA5.

In dit proefschrift is de focus gericht op twee belangrijke aspecten: I) het vergroten van onze kennis over het werkingsmechanisme van ANXA5-gebaseerde antistolling op PS bevattende celmembranen; II) het verkrijgen van meer inzicht in de biologische significantie van endogeen ANXA5.

ANXA5 lattices interfereren in de tenase complexvorming op PS-exposerende celoppervlakken

De PS bevattende celmembraan is een sterke katalysator van de bloedstolling. De antistollende activiteiten van annexines zijn gebaseerd op het afschermen van deze stollingsbevorderende celoppervlakken. ANXA5 heeft van alle annexines de hoogste affiniteit voor negatief geladen fosfolipiden en in theorie ook de hoogste antistollende activiteit. In **hoofdstuk 2** hebben we hiervoor een verklaring gezocht door de structurele organisatie van ANXA5 op model membranen en op PS bevattende celmembranen nader te onderzoeken. Hiervoor werd een flowcytometrische en confocale FRET (Fluorescence Resonance Energy Transfer) methode gebruikt om ANXA5 interacties te visualiseren. Met deze aanpak werd duidelijk aangetoond dat ANXA5, na binding aan ionomycine-geactiveerde bloedplaatjes en ionomycine-gestimuleerde COS1 cellen, een hoog FRET signaal vertoonde wat een aanwijzing is voor membraangebonden gestructureerde arrays. We toonden ook aan dat de oppervlak-geassocieerde ANXA5 organisatie gebaseerd is op meervoudige ANXA5 interacties (two-step FRET) en dat deze ANXA5-ANXA5 interacties 2-dimensionaal zijn (d.w.z., één laagje dik). Deze interacties worden ook wel aangeduid als 2D-lattices. Verder gebruikten we dezelfde FRET gebaseerde experimentele aanpak om de interacties tussen FIX en FVIII van de intrinsieke route van de stollingscascade te visualiseren. FIX-FVIII interacties werden geremd door lage concentraties (5 nM) van pre-gebonden ANXA5. ANXA1, dat ook aan PS bindt maar geen 2D lattices vormt, remde gedeeltelijk de FIX-FVIII interacties. Uit deze waarnemingen hebben we geconcludeerd dat, naast de afscherming van negatief geladen fosfolipiden, de organisatie van ANXA5 op PS-exposerende celoppervlakken ook relevant is voor de ANXA5-gebaseerde antistolling. Onze werkhypothese is dat de mobiliteit van PS-gebonden FIX en FVIII in aanwezigheid van ANXA5 arrays zodanig beperkt is dat er geen interacties tussen stollingsfactoren kunnen plaatsvinden en er geen tenase complex gevormd kan worden. Ondanks dat we de mobiliteit van de stollingsfactoren niet direct hebben kunnen meten hebben we gegronde redenen om aan te nemen dat ANXA5 arrays interfereren in de tenase complexvorming (en waarschijnlijk ook in de complexvorming van andere stollingsfactoren). Door deze waarnemingen kunnen we concluderen dat het afschermen van PS-moleculen en het verminderen van de laterale mobiliteit van PS-gebonden stollingsfactoren belangrijke onderdelen zijn van de antistollende activiteit van ANXA5.

Genetische variaties in het *ANXA5* gen en ANXA5 plasmaspiegels
Genetische variaties komen in alle genen van ons lichaam voor. Genetische variaties kunnen verantwoordelijk zijn voor het ontstaan van een bepaalde ziekte of bijdragen aan de pathofysiologie van een ziekte. In het *ANXA5* gen zijn ook verschillende genetische variaties gevonden. In **hoofdstuk 3** zijn single-nucleotide polymorfismen (SNPs) en veelvoorkomende haplotypen (d.w.z., een combinatie van genetische variaties; haplotype frequentie >1%) binnen het *ANXA5* promoter gebied beschreven en is een associatie van genetische variaties met ANXA5 plasmaspiegels in gezonde personen bestudeerd. In vergelijking met de drie *ANXA5* promoter haplotypes die eerder beschreven werden door *Bogdanova et al.*, identificeerden wij vier veelvoorkomende haplotypen H1-H4 in het *ANXA5* promoter gebied. Tevens bleek uit onze studie dat de haplotypen een effect hadden op de ANXA5 plasmaspiegels. Zo is haplotype H2 geassocieerd met lagere plasma ANXA5 levels en haplotype H4 (tagged door SNP rs62319820) – met hogere ANXA5 plasmaspiegels. We hebben bovendien aangetoond dat Bogdanova's wilde-type haplotype N uit twee haplotypen (H1 en H2) bestaat.

Onze bevinding dat het *ANXA5* haplotype H2 en H4 geassocieerd is met respectievelijk lagere en hogere ANXA5 plasmaspiegels werd verder bevestigd in de patiëntengroep met atherosclerose (**hoofdstuk 5**). Echter bleken de plasma ANXA5 levels niet van klinisch belang te zijn, omdat er geen enkele correlatie werd gevonden met de klinische uitkomstparameters, zoals de dikte van de vaatwand (IMT), bloed cholesterol spiegels en een ontsteking marker (hs-CRP)). Bovendien waren plasma ANXA5 levels niet geassocieerd met trombose of herhaalde miskramen bij patiënten met auto-immuunziekten (**hoofdstuk 6**).

De relevantie van *ANXA5* haplotypes in (cardio)vasculaire ziektebeelden
Atherosclerose (in de volksmond ook aderverkalking genoemd) is een ziektebeeld waarin zowel de ontsteking (ook wel aangeduid als inflammatie) als ook de stolling een rol spelen. ANXA5 heeft antistollende activiteiten en anti-inflammatoire eigenschappen. In **hoofdstuk 4** hebben we onderzocht of *ANXA5* genetische variaties (d.w.z., individuele SNPs en haplotypen H1-H4) als biomarkers bij patiënten met atherosclerose zouden kunnen dienen. Dat hebben we gedaan bij patiënten met familiaire hypercholesterolemie (FH), een ziekte die zich gekenmerkt door de hoge LDL-cholesterol ("slecht cholesterol") spiegels in het bloed en een verhoogd risico op vroegtijdige hart- en vaatziekten. Bij FH patiënten uit de ASAP trial (Atorvastatine versus Simvastatine op Atherosclerose Progressie) hebben we een associatie van *ANXA5* genetische varianten en / of ANXA5 plasmaspiegels met IMT (d.w.z., de dikte van de vaatwand, een marker van atherosclerose) onderzocht. In een tweede grote groep van FH patiënten uit de GIRaFH (Genetische Identificatie van Risicofactoren bij Familiaire Hypercholesterolemie) studie bestudeerden we bovendien of de variaties in het *ANXA5* gen bijdragen aan het risico op hart- en vaatziekten. Onze resultaten lieten zien dat haplotypen H1-H4 en de individuele SNPs niet geassocieerd zijn met de

IMT progressie (d.w.z., progressie van atherosclerose). Ook bleken de ANXA5 plasmaspiegels niet geassocieerd met de IMT parameters. Geen van de onderzochte genetische variaties leverden een bijdrage aan een verhoogd cardiovasculair risico. Uit de hierboven beschreven data kunnen we concluderen dat ANXA5 als biomarker geen voorspellende waarde heeft bij atherosclerose; in ieder geval niet bij de patiënten met FH.

In **hoofdstuk 5** hebben we een associatie tussen *ANXA5* promoter SNPs / veelvoorkomende haplotypen H1-H4 en het risico op diepe veneuze trombose (DVT) onderzocht in de Nederlandse bevolking. We hebben geen enkele associatie gevonden tussen individuele promoter SNPs of haplotypen H1-H4 en het DVT risico. De frequenties van genotypes en haplotypen waren gelijk verdeeld zowel tussen patiënten met nieuwe gediagnosticeerde DVT en ziekenhuis controles zonder voorafgaande veneuze trombo-embolie (Amsterdam Case-control Trombofilie (ACT) studie) als ook tussen DVT patiënten en een grote controlegroep uit de bevolking (Nijmegen Biomedische Studie, NBS). Onze studie had voldoende power (~80%) om genotype- en/of haplotype-specifieke verschillen te detecteren. Daarom hebben we geconcludeerd dat *ANXA5* genetische varianten niet geassocieerd zijn met het DVT risico in de Nederlandse bevolking.

Er is voldoende bewijs voor de betrokkenheid van ANXA5 in het antifosfolipidensyndroom (APS). APS is een auto-immuunziekte die wordt gekarakteriseerd door de persistente aanwezigheid van antifosfolipide antistoffen in plasma van patiënten met een voorgeschiedenis van vasculaire trombose en / of herhaalde miskramen. Of en in welke mate genetische variaties in *ANXA5* het risico op trombose of zwangerschapscomplicaties bij APS patiënten verhogen is in **hoofdstuk 6** onderzocht. Allereerst hebben we aangetoond dat *ANXA5* haplotypen H1-H4 niet zonder meer geassocieerd zijn met een verhoogde kans op trombose bij deze patiënten. Wel hebben we laten zien dat *ANXA5* H3 vaker voorkwam bij vrouwen met een obstetrisch APS ten opzichte van een grote controlegroep. *ANXA5* haplotypen H1-H4 waren niet geassocieerd met een verhoogde productie van antifosfolipide antistoffen noch met ANXA5 plasmaspiegels. Of de biomarker *ANXA5* H3 bij vrouwen met een obstetrisch APS daadwerkelijk een veelbelovende marker voor zwangerschap gerelateerde complicaties is, zal in de toekomst verder uitgezocht moeten worden.

In **hoofdstuk 7** worden de conclusies van de verschillende hoofdstukken in perspectief geplaatst en uitgebreid bediscussieerd. Naar aanleiding van de bevindingen van dit proefschrift worden een aantal suggesties besproken voor vervolgonderzoek.

DANKWOORD

Ik begin met de woorden van Vladimir Majakovski (een Russische dichter) uit zijn werk "Een bijzonder avontuur" (1920):

"Светить всегда, светить везде, До дней последних донца, Светить - и никаких гвоздей! Вот лозунг мой и солнца!"	Schijnen, altijd en overal, tot de laatste dagen, schijnen, en verder niemandal Dat is ons beider (van mij en van de zon) Motto!

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UNIT TROMBOSE EN HEMOSTASE

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MARIEKE ***ROBERT*** ***BAS***

CURRICULUM VITAE

Larissa Emelianova werd op 19 januari 1968 geboren in Izhevsk, de hoofdstad van de autonome republiek Oedmoertië (Russisch: Удму́ртия) in de Russische Federatie. Ze heeft kindergeneeskunde gestudeerd aan de Medische Staatsacademie te Izhevsk (diploma cum laude). Na het voltooien van haar opleiding ging ze werken als kinderarts in het klinisch kinderziekenhuis te Izhevsk. Bovendien was ze verbonden aan de faculteit Pediatrie van de Medische Staatsacademie als docent kinderziekten. Ze deed onderzoek naar de chronische longaandoeningen bij kinderen, waaronder cystic fibrosis en bronchiale astma. In juni 1999 trouwde ze met Bennie Hiddink uit Baak (Gelderland). In december 1999 promoveerde ze op het proefschrift 'De klinisch-metabolische bijzonderheden van bronchiale astma bij kinderen" en emigreerde naar Nederland.

In Nederland heeft ze een nieuwe start gemaakt door het afronden van de opleiding "Biologie en Medisch Laboratoriumonderzoek" aan de Saxion Hogeschool te Deventer (diploma cum laude). Sinds eind 2006 werkte ze in verschillende functies bij het laboratorium hematologie (unit trombose en hemostase) van het Radboudumc: stagiaire, research analist en promovenda. In januari 2009 begon ze aan haar promotietraject onder begeleiding van prof. dr. T.M. de Witte, prof. dr. J.H. Jansen en dr. W.L. van Heerde. De resultaten van dit onderzoek staan beschreven in dit proefschrift.

Van september 2013 tot maart 2014 heeft ze op vrijwillige basis gewerkt aan een tumor microparticles project bij prof. dr. L.W. Terstappen (Universiteit Twente, Enschede). Sinds april 2014 is de auteur van dit proefschrift werkzaam als datamanager trialbureau bij het Integraal Kankercentrum Nederland. Ze houdt zich bezig met het lokale datamanagement van hematologische en oncologische studies.

LIST OF PUBLICATIONS

Hiddink L., Jacobs N., Woestenenk R., Fransen J.A.M., Brachvogel B., Loof A., Boezeman J., van Heerde W.L. Annexin A5 membrane-bound lattices interfere in tenase complex formation on phosphatidylserine-exposing cells: evidence from a Fluorescence Resonance Energy Transfer study *Submitted.*

de Jong P.G., **Hiddink L.**, Meijers J.C.M., Kiemeney L.A., van Heerde W.L., and Middeldorp S. No association between *Annexin A5* genetic variants and deep venous thrombosis *British Journal of Haematology 2015; 169: 301-304.*

Hiddink L., de Laat B., Derksen R.H.W.M., de Groot P.G., van Heerde W.L. *Annexin A5* haplotypes in the antiphospholipid syndrome *Thrombosis Research 2015; 135: 417-419.*

Hiddink L., Dallinga-Thie G.M., Hovingh G.K., de Visser M.C.H., Peer P.G.M., Stalenhoef A.F.H., van Heerde W.L. *Annexin A5* haplotypes in familial hypercholesterolemia: lack of association with carotid intima-media thickness and cardiovascular disease risk *Atherosclerosis 2015; 238: 195-200.*

Hiddink L., de Visser M.C.H., van Heerde W.L. Polymorphisms in the *Annexin A5* gene influence circulating Annexin A5 levels in healthy controls *Thrombosis Research 2012; 129: 815-817.*

Emelianova L. (1999) Thesis "The clinical and metabolic characteristics of children's bronchial asthma" (in Russian).